

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
7 September 2001 (07.09.2001)

PCT

(10) International Publication Number
WO 01/64681 A2

(51) International Patent Classification⁷: C07D 491/04,
471/04, A61K 31/437, 31/407, A61P 35/00

(21) International Application Number: PCT/US01/06214

(22) International Filing Date: 28 February 2001 (28.02.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/185,536 28 February 2000 (28.02.2000) US

(63) Related by continuation (CON) or continuation-in-part
(CIP) to earlier application:
US 60/185,536 (CIP)
Filed on 28 February 2000 (28.02.2000)

(71) Applicant (for all designated States except US): SUGEN,
INC. [US/US]; 230 E. Grand Avenue, South San Francisco,
CA 94080-4811 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): TANG, Peng, Cho
[US/US]; 827 Camino Ricardo, Moraga, CA 94556 (US).
MILLER, Todd, A. [US/US]; 1713 N.E. Hollow Tree
Lane, Bend, OR 97701 (US). LI, Xiaoyuan [US/US];
2020 Victoria Court, Los Altos, CA 94024 (US). ZHANG,
Ruofei [CN/US]; 1046 Gull Avenue, Foster City, CA

94404 (US). CUI, Jingrong [CN/US]; 755 Ohlone Av-
enue, #764, Albany, CA 94706 (US). HUANG, Ping
[CN/US]; 117 Whit's Road, Mountain View, CA 94040
(US). WEI, Chung, Chen [US/US]; 39 Commons Lane,
Foster City, CA 94404 (US).

(74) Agents: BURROUS, Beth, A. et al.; Foley & Lardner,
3000 K. Street, N.W., Suite 500, Washington, DC 20007-
5109 (US).

(81) Designated States (national): AE, AL, AM, AT, AU, AZ,
BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE,
ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP,
KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD,
MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD,
SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ,
VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,
CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished
upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

WO 01/64681 A2

(54) Title: 3-(PYROLYLLACTONE)-2-INDOLINONE COMPOUNDS AS KINASE INHIBITORS

(57) Abstract: The invention relates to certain indolinone compounds, their method of synthesis, and a combinatorial library con-
sisting of the indolinone compounds of the invention. The invention also relates to methods of modulating the function of protein
kinases using indolinone compounds of the invention and methods of treating diseases by modulating the function of protein kinases
and related signal transduction pathways.

3-(PYROLYLLACTONE)- 2-INDOLINONE COMPOUNDS AS KINASE INHIBITORS

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CROSS-REFERENCE

This application claims priority under 35 U.S.C. 119(e) to U.S. Provisional Application Serial No. 60/185,536, filed on February 28, 2000, the disclosure of which is herein incorporated by reference in its entirety.

10 Field of Invention

The invention relates to certain indolinone compounds, their method of synthesis, and a combinatorial library consisting of the indolinone compounds of the invention. The invention also relates to methods of modulating the function of protein kinases, in particular CDK2 protein kinase, using compounds of the invention and methods of treating diseases by modulating the function of protein
15 kinases, in particular CDK2 protein kinase, and related signal transduction pathways.

State of the Art

The following description of the background of the invention is provided to
20 aid in understanding the invention, but is not admitted to describe or constitute prior art to the invention.

Cellular signal transduction is a fundamental mechanism whereby extracellular stimuli are relayed to the interior of cells and subsequently regulate diverse cellular processes. One of the key biochemical mechanisms of signal
25 transduction involves the reversible phosphorylation of proteins. Phosphorylation of polypeptides regulates the activity of mature proteins by altering their structure and function. Phosphate most often resides on the hydroxyl moiety (-OH) of serine, threonine, or tyrosine amino acids in proteins.

Enzymes that mediate phosphorylation of cellular effectors generally fall into
30 two classes. The first class consists of protein kinases which transfer a phosphate moiety from adenosine triphosphate to protein substrates. The second class consists

of protein phosphatases which hydrolyze phosphate moieties from phosphoryl protein substrates. The converse functions of protein kinases and protein phosphatases balance and regulate the flow of signals in signal transduction processes.

5 Protein kinases and protein phosphatases are generally divided into two groups - receptor and non-receptor type proteins. Most receptor-type protein phosphatases contain two conserved catalytic domains, each of which encompasses a segment of 240 amino acid residues. Saito *et al.*, 1991, *Cell Growth and Diff.* 2:59-65. Receptor protein phosphatases can be subclassified further based upon the
10 amino acid sequence diversity of their extracellular domains. Saito *et al.*, *supra*; Krueger *et al.*, 1992, *Proc. Natl. Acad. Sci. USA* 89:7417-7421.

Protein kinases and protein phosphatases are also typically divided into three classes based upon the amino acids they act upon. Some catalyze the addition or hydrolysis of phosphate on serine or threonine only, some catalyze the addition or
15 hydrolysis of phosphate on tyrosine only, and some catalyze the addition or hydrolysis of phosphate on serine, threonine, and tyrosine.

Kinases can regulate the catalytic activity of other protein kinases involved in cell proliferation. Protein kinases with inappropriate activity are also involved in some types of cancer. Abnormally elevated levels of cell proliferation are associated
20 with receptor and non-receptor protein kinases with unregulated activity.

In addition to their role in cellular proliferation, protein kinases are thought to be involved in cellular differentiation processes. Cell differentiation occurs in some cells upon nerve growth factor (NGF) or epidermal growth factor (EGF) stimulation. Cellular differentiation is characterized by rapid membrane ruffling,
25 cell flattening, and increases in cell adhesion. Chao, 1992, *Cell* 68:995-997.

In an effort to discover novel treatments for cancer and other diseases, biomedical researchers and chemists have designed, synthesized, and tested molecules that inhibit the function of protein kinases. Some small organic molecules form a class of compounds that modulate the function of protein kinases.
30 Examples of molecules that have been reported to inhibit the function of protein kinases are bis-monocyclic, bicyclic or heterocyclic aryl compounds (PCT WO

92/20642), vinylene-azaindole derivatives (PCT WO 94/14808), 1-cyclopropyl-4-pyridyl-quinolones (U.S. Patent No. 5,330,992), styryl compounds (by Levitzki, et al., U.S. Patent No. 5,217,999, and entitled "Styryl Compounds which Inhibit EGF Receptor Protein Tyrosine Kinase), styryl-substituted pyridyl compounds (U.S. Patent No. 5,302,606), certain quinazoline derivatives (EP Application No. 0 566 266 A1), seleoindoles and selenides (PCT WO 94/03427), tricyclic polyhydroxylic compounds (PCT WO 92/21660), and benzylphosphonic acid compounds (PCT WO 91/15495).

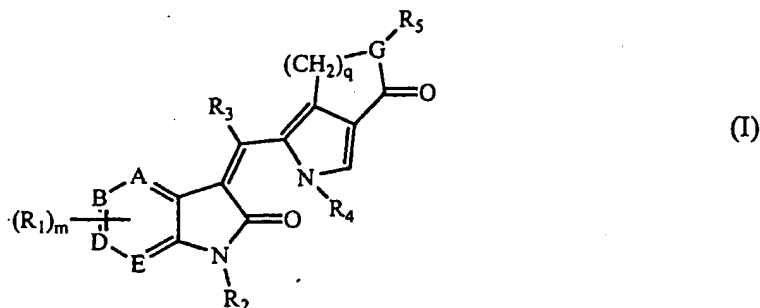
The compounds that can traverse cell membranes and are resistant to acid hydrolysis are potentially advantageous therapeutics as they can become highly bioavailable after being administered orally to patients. However, many of these protein kinase inhibitors only weakly inhibit the function of protein kinases. In addition, many inhibit a variety of protein kinases and will therefore cause multiple side-effects as therapeutics for diseases.

Despite the significant progress that has been made in developing compounds for the treatment of cancer, there remains a need in the art to identify the particular structures and substitution patterns that form the compounds capable of modulating the function of particular protein kinases. The present invention fulfils this need.

SUMMARY OF THE INVENTION

The present invention is directed to certain indolinone compounds and methods of treating diseases mediated by protein kinases, in particular CDK2 protein kinase, using these compounds.

Thus, in a first aspect, the invention provides a compound of Formula (I):



wherein:

- (a) each R_i is independently and optionally selected from the group consisting of:
- (i) hydrogen;
 - (ii) saturated or unsaturated alkyl optionally substituted with one, two or three substituents selected from the group consisting of halogen, trihalomethyl, alkoxy, carboxylate, amino, nitro, ester, and a five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where each ring is optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;
 - (iii) an aromatic or heteroaromatic ring optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;
 - (iv) an aliphatic or heteroaliphatic ring optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, ester, and an aromatic or heteroaromatic ring optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;
 - (v) an alcohol of formula $-(X_1)_{n1}-OH$ or an alkoxyalkyl of formula $-(X_2)_{n2}-O-X_3$, where X_1 , X_2 , and X_3 are independently selected from the group consisting of saturated or unsaturated alkyl, amide, and five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where the alkyl, and each ring is optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, alkoxy, aryl, heteroaryl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester; and $n1$ and $n2$ are independently 0 or 1;
 - (vi) a halogen or trihalomethyl;
 - (vii) a carboxylic acid of formula $-(X_4)_{n4}-COOH$ or ester of formula $-(X_5)_{n5}-COO-X_6$, where X_4 , X_5 , and X_6 are independently selected from the group consisting of alkyl and five-membered or six-membered aromatic, heteroaromatic, aliphatic, or

heteroaliphatic ring; and n4 and n5 are independently 0 or 1;

(viii) an amide or thioamide of formula $-(X_7)_{n7}-NHCOX_8$, $-(X_7)_{n7}-NHCSX_8$, $-(X_9)_{n9}-CONX_{10}X_{11}$, or of formula $-(X_9)_{n9}-CSNX_{10}X_{11}$, where X_7 and X_9 are each independently selected from the group consisting of alkyl and five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where each of

the ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester; n7 and n9 are independently 0 or 1; and X_8 , X_{10} , and X_{11} are each independently selected from the group consisting of hydrogen, alkyl, hydroxyl, alkoxy, and five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where the ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, thioalkyl, aryl, heteroaryl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester; or where X_{10} and X_{11} taken together form a five-membered or six-membered aliphatic or heteroaliphatic ring or a fused bicyclic ring optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;

(ix) a sulfonamide of formula $-(X_{12})_{n12}-SO_2NX_{13}X_{14}$ or of formula $-(X_{12})_{n12}-NX_{13}-SO_2-X_{14}$, where X_{12} is selected from the group consisting of alkyl, and five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where the alkyl and each ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, halogen, hydroxy, trihalomethyl, carboxylate, amino, nitro, and ester; n12 is 0, 1, or 2; and X_{13} , and X_{14} are independently selected from the group consisting of hydrogen, alkyl, and five-, or six-membered aromatic, heteroaromatic, three-, four-, five-, or six-membered aliphatic, or heteroaliphatic ring where the alkyl and each ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, aryl, heteroaryl, halogen, trihalomethyl, aldehyde, carboxylate, amino, nitro, and ester; or where X_{13} and X_{14} taken together form a five-membered or six-membered aliphatic or heteroaliphatic

- ring or a fused bicyclic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;
- (x) a sulfone of formula $-(X_{15})_{n15}-SO_3H$, where X_{15} is selected from the group consisting of alkyl, and five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where the alkyl and each ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester; $n15$ is 0, 1, or 2;
- (xi) an amine of formula $-(X_{16})_{n16}-NX_{17}X_{18}$, where X_{16} is selected from the group consisting of saturated or unsaturated alkyl, and five-membered or six-membered aromatic, heteroaromatic, or aliphatic ring; $n16$ is 0 or 1; and X_{17} and X_{18} are independently selected from the group consisting of hydrogen, saturated or unsaturated alkyl, and five-membered or six-membered aromatic, heteroaromatic, or aliphatic ring where each ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;
- (xii) a ketone of formula $-(X_{19})_{n19}-CO-X_{20}$, where X_{19} and X_{20} are independently selected from the group consisting of alkyl and five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where the alkyl and each ring is optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring, halogen, trihalomethyl, carboxylate, amino, nitro, and ester; and $n19$ is 0 or 1;
- (xiii) a nitro group of the formula $-NO_2$; or
- (xiv) two adjacent R_1 groups together along with two adjacent A, B, D, or E form an optionally substituted five-membered or six-membered aliphatic or heteroaliphatic ring;
- (b) R_2 and R_4 are each independently selected from the group consisting of
- (i) hydrogen;
- (ii) saturated or unsaturated alkyl optionally substituted with one, two or three

- substituents selected from the group consisting of halogen, trihalomethyl, carboxylate, amino, nitro, ester, and a five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where each ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;
- (iii) an aromatic or heteroaromatic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, and ester; and
- (iv) an aliphatic or heteroaliphatic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, ester, and an aromatic or heteroaromatic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;
- (c) R_3 and R_4 are each independently selected from the group consisting of
- (i) hydrogen;
- (ii) saturated or unsaturated alkyl optionally substituted with one, two or three substituents selected from the group consisting of hydroxy, halogen, trihalomethyl, carboxylate, amino, nitro, ester, and a five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where each ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;
- (iii) an aromatic or heteroaromatic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, and ester; and
- (iv) an aliphatic or heteroaliphatic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, ester, and an aromatic or heteroaromatic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;

(d) A, B, D, and E are each independently carbon or nitrogen provided that none, one, or, two of A, B, D, and E is nitrogen; and provided that when any of A, B, D, or E is nitrogen, no R₁ is attached to said A, B, D, or E;

(e) G is nitrogen or oxygen, with the proviso that when G is oxygen, R₂ is absent;

(f) m is 2, 3, or 4; and

(g) q is 1, 2, 3, or 4; or

a pharmaceutically acceptable salt thereof.

In a second aspect this invention is directed to a pharmaceutical composition comprising one or more compound(s) of Formula (I) or a pharmaceutically acceptable salt thereof and a pharmaceutically acceptable excipient.

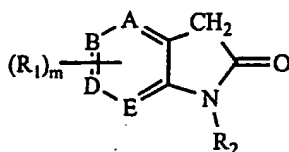
In a third aspect, this invention is directed to a method of treating diseases mediated by abnormal protein kinase activity, in particular CDK2 protein kinase, in an organism, in particular humans, which method comprises administering to said organism a pharmaceutical composition comprising a compound of Formula (I). Such diseases include by way of example and not limitation, cancer, diabetes, hepatic cirrhosis, cardiovascular disease such as atherosclerosis, angiogenesis, immunological disease such as autoimmune disease and renal disease. Though not limited to any particular protein kinase, it is believed that the compounds of the invention are selective for CDK2 protein kinase over other kinases such as FLK, FGFR, EGFR, PDGFR, and SRC.

In a fourth aspect, this invention is directed to a method of modulating of the catalytic activity of PKs, in particular, receptor tyrosine kinases (RTKs), non-receptor protein tyrosine kinases (CTKs) and serine/threonine protein kinases (STKs), using a compound of this invention which may be carried out in vitro or in vivo. In particular, the receptor protein kinase whose catalytic activity is modulated by a compound of this invention is selected from the group consisting of EGF, HER2, HER3, HER4, IR, IGF-1R, IRR, PDGFR α , PDGFR β , CSFIR, C-Kit, C-fms, Flk-1R, Flk4, KDR/Flk-1, Flt-1, FGFR-1R, FGFR-2R, FGFR-3R and FGFR-4R. The cellular tyrosine kinase whose catalytic activity is modulated by a compound of this invention is selected from the group consisting of Src, Frk, Btk, Csk, Abl,

ZAP70, Fes/Fps, Fak, Jak, Ack, Yes, Fyn, Lyn, Lck, Blk, Hck, Fgr and Yrk. The serine-threonine protein kinase whose catalytic activity is modulated by a compound of this invention is selected from the group consisting of CDK2 and Raf.

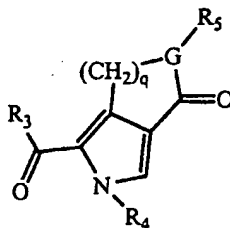
In a fifth aspect, this invention is directed to the use of a compound of
 5 Formula (I) in the preparation of a medicament which is useful in the treatment of a disease mediated by abnormal protein kinase activity.

In a sixth aspect, the invention provides a combinatorial library of at least 10 indolinone compounds that can be formed by reacting an oxindole with an aldehyde or a ketone, where the oxindole has the following structure:



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and the aldehyde or ketone has the following structure:



and R₁, R₃, R₄, R₅, A, B, D, E, G, m, and q are as described herein.

Lastly, this invention is also directed to a method of identifying a chemical
 15 compound that modulates the catalytic activity of a protein kinase by contacting cells expressing said protein kinase with said compound and then monitoring said cells for an effect.

DETAILED DESCRIPTION OF THE INVENTION

20

Definitions

Unless otherwise stated the following terms used in the specification and claims have the meanings discussed below:

As used herein, the term "alkyl" refers to an aliphatic hydrocarbon group. The alkyl moiety may be a "saturated alkyl" group, which means that it does not

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contain any alkene or alkyne moieties. The alkyl moiety may also be an "unsaturated alkyl" moiety, which means that it contains at least one alkene or alkyne moiety. An "alkene" moiety refers to a group consisting of at least two carbon atoms and at least one carbon-carbon double bond, and an "alkyne" moiety
5 refers to a group consisting of at least two carbon atoms and at least one carbon-carbon triple bond. The alkyl moiety, whether saturated or unsaturated, may be branched, non-branched, or cyclic.

The alkyl group has 1 to 20 carbon atoms (whenever it appears herein, a numerical range such as "1 to 20" refers to each integer in the given range; e.g., "1 to
10 20 carbon atoms" means that the alkyl group may consist of 1 carbon atom, 2 carbon atoms, 3 carbon atoms, etc., up to and including 20 carbon atoms, although the present definition also covers the occurrence of the term "alkyl" where no numerical range is designated). More preferably, it is a "medium" size alkyl having 1 to 10 carbon atoms. Most preferably, it is a "lower" alkyl having 1 to 4 carbon atoms e.g.,
15 methyl, ethyl, propyl, isopropyl, n-butyl, tert-butyl, iso-butyl. The alkyl group may be substituted or unsubstituted. When substituted, the substituent group(s) is(are) preferably one or more group(s) individually and independently selected from hydroxy, alkoxy, mercapto, alkylthio, cyano, halo, carbonyl, nitro, and amino.

The term "aromatic" refers to a mono or bicyclic aromatic group of 6 to 12
20 carbon atoms which has at least one ring having a conjugated pi electron system e.g., phenyl, naphthyl or anthracene groups.

The term "heteroaromatic" refers to a mono or bicyclic aromatic group of 5 to 10 ring atoms wherein one, two or three rings atoms are selected from a group consisting of nitrogen, oxygen or sulfur, the remaining ring atoms being carbon e.g.,
25 pyridine, pyrrole, thiophene, indole, imidazole, quinoline, isoquinoline, pyrazine, furan, pyrimidine, oxazole, triazole, pyrazole and the like.

The term "aliphatic ring" refers to a saturated cyclic group of 3 to 10 carbon atoms e.g., cyclopropane, cyclobutane, cyclopentane, cyclohexane and the like.

The term "heteroaliphatic ring" refers to a saturated cyclic group of 5 to 10
30 ring atoms wherein one, two or three ring atoms are selected from the group consisting of nitrogen, sulfur, sulfoxide, sulfone, $-\text{SO}_2\text{O}-$ group or oxygen the

remaining ring atoms being carbon *e.g.*, tetrahydropyran, piperidine, pyrrolidine, piperazine, morpholine and the like.

The term "amine" refers to a chemical moiety of formula $-NR_aR_b$, where R_a and R_b are independently selected from the group consisting of hydrogen, saturated or unsaturated alkyl, and five-membered or six-membered aromatic or heteroaromatic ring, where the ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, nitro, and ester moieties.

The term "halogen" refers to an atom selected from the group consisting of fluorine, chlorine, bromine, and iodine.

The term "trihalomethyl" refers to the $-CX_3$ group, where X is a halogen, *e.g.*, trifluoromethyl.

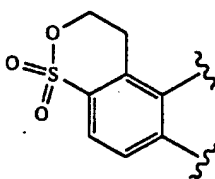
The term "carboxylic acid" or "carboxylate" refers to a chemical moiety with formula $-(R)_n-COOH$, where R is selected from the group consisting of saturated or unsaturated alkyl and five-membered or six-membered aromatic or heteroaromatic ring and where n is 0 or 1.

The term "ester" refers to a chemical moiety with formula $-(R)_n-COOR'$, where R and R' are independently selected from the group consisting of saturated or unsaturated alkyl and five-membered or six-membered aromatic or heteroaromatic ring and where n is 0 or 1.

The term "aldehyde" refers to a chemical moiety with formula $-(R)_n-CHO$, where R is selected from the group consisting of saturated or unsaturated alkyl and five-membered or six-membered aromatic or heteroaromatic ring and where n is 0 or 1.

The term "alkoxy" refers to a group $-OR_c$ where R_c is unsubstituted lower alkyl *e.g.*, methoxy, ethoxy, propoxy, butoxy, and the like.

The term "fused bicyclic ring" refers to a five- or six-membered heteroaliphatic ring fused with an aromatic or heteroaromatic ring. A non-limiting example of a fused bicyclic ring is:



"Optional" or "optionally" means that the subsequently described event or circumstance may but need not occur, and that the description includes instances where the event or circumstance occurs and instances in which it does not. For example, "alkyl group optionally substituted with halogen" means that the halogen may, but need not be present, and the description includes situations where the alkyl group is substituted with a halogen group and situations where the alkyl group is not substituted with the halogen group.

Compounds that have the same molecular formula but differ in the nature or sequence of bonding of their atoms or the arrangement of their atoms in space are termed "isomers". Isomers that differ in the arrangement of their atoms in space are termed "stereoisomers". Stereoisomers that are not mirror images of one another are termed "diastereomers" and those that are non-superimposable mirror images of each other are termed "enantiomers". When a compound has an asymmetric center, for example, it is bonded to four different groups, a pair of enantiomers is possible. An enantiomer can be characterized by the absolute configuration of its asymmetric center and is described by the R- and S-sequencing rules of Cahn and Prelog, or by the manner in which the molecule rotates the plane of polarized light and designated as dextrorotatory or levorotatory (i.e., as (+) or (-)-isomers respectively). A chiral compound can exist as either individual enantiomer or as a mixture thereof. A mixture containing equal proportions of the enantiomers is called a "racemic mixture".

The compounds of this invention may possess one or more asymmetric centers; such compounds can therefore be produced as individual (R)- or (S)- stereoisomers or as mixtures thereof. For example, if the R¹ substituent in a compound of Formula (I) is 2-methoxyethyl, then the carbon to which the methoxy group is attached is an asymmetric center and therefore the compound of Formula (I) can exist as an (R)- or (S)-stereoisomer. Unless indicated otherwise, the description or naming of a particular compound in the specification and claims is intended to

include both individual enantiomers and mixtures, racemic or otherwise, thereof. The methods for the determination of stereochemistry and the separation of stereoisomers are well-known in the art (*see* discussion in Chapter 4 of "Advanced Organic Chemistry," 4th edition J. March, John Wiley and Sons, New York, 1992).

5 The compounds of Formula (I) may exhibit the phenomena of tautomerism and structural isomerism. For example, the compounds described herein may adopt an E or a Z configuration about the double bond connecting the 2-indolinone moiety to the pyrrole moiety or they may be a mixture of E and Z. This invention encompasses any tautomeric or structural isomeric form and mixtures thereof which
10 possess the ability to modulate RTK, CTK and/or STK activity and is not limited to any one tautomeric or structural isomeric form.

 A "pharmaceutical composition" refers to a mixture of one or more of the compounds described herein, or physiologically/pharmaceutically acceptable salts or prodrugs thereof, with other chemical components, such as
15 physiologically/pharmaceutically acceptable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

 The compound of Formula (I) may also act as a prodrug. A "prodrug" refers to an agent which is converted into the parent drug in vivo. Prodrugs are often
20 useful because, in some situations, they may be easier to administer than the parent drug. They may, for instance, be bioavailable by oral administration whereas the parent drug is not. The prodrug may also have improved solubility in pharmaceutical compositions over the parent drug. An example, without limitation, of a prodrug would be a compound of the present invention which is administered as an ester (the
25 "prodrug") to facilitate transmittal across a cell membrane where water solubility is detrimental to mobility but then is metabolically hydrolyzed to the carboxylic acid, the active entity, once inside the cell where water solubility is beneficial.

 A further example of a prodrug might be a short polypeptide, for example, without limitation, a 2 - 10 amino acid polypeptide, bonded through a terminal
30 amino group to a carboxy group of a compound of this invention wherein the

polypeptide is hydrolyzed or metabolized in vivo to release the active molecule. The prodrugs of a compound of Formula (I) are within the scope of this invention.

Additionally, it is contemplated that a compound of Formula (I) would be metabolized by enzymes in the body of the organism such as human being to
5 generate a metabolite that can modulate the activity of the protein kinases. Such metabolites are within the scope of the present invention.

A "combinatorial library" refers to all the compounds formed by the reaction of each compound of one dimension with a compound in each of the other dimensions in a multi-dimensional array of compounds. In the context of the present
10 invention, the array is two dimensional and one dimension represents all the oxindoles of the invention and the second dimension represents all the aldehydes of the invention. Each oxindole may be reacted with each and every aldehyde in order to form an indolinone compound. All indolinone compounds formed in this way are within the scope of the present invention. Also within the scope of the present
15 invention are smaller combinatorial libraries formed by the reaction of some of the oxindoles with all of the aldehydes, all of the oxindoles with some of the aldehydes, or some of the oxindoles with some of the aldehydes.

As used herein, a "pharmaceutically acceptable carrier" refers to a carrier or diluent that does not cause significant irritation to an organism and does not abrogate
20 the biological activity and properties of the administered compound.

An "pharmaceutically acceptable excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of a compound. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin,
25 vegetable oils and polyethylene glycols.

As used herein, the term "pharmaceutically acceptable salt" refers to those salts which retain the biological effectiveness and properties of the parent compound. Such salts include:

(i) acid addition salt which is obtained by reaction of the free base of the
30 parent compound with inorganic acids such as hydrochloric acid, hydrobromic acid, nitric acid, phosphoric acid, sulfuric acid, and perchloric acid and the like, or with

organic acids such as acetic acid, oxalic acid, (D) or (L) malic acid, maleic acid, methanesulfonic acid, ethanesulfonic acid, *p*-toluenesulfonic acid, salicylic acid, tartaric acid, citric acid, succinic acid or malonic acid and the like, preferably hydrochloric acid or (L)-malic acid such as the L-malate salt of 5-(5-fluoro-2-oxo-
5 1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid(2-diethylaminoethyl)amide; or

(2) salts formed when an acidic proton present in the parent compound either is replaced by a metal ion, e.g., an alkali metal ion, an alkaline earth ion, or an aluminum ion; or coordinates with an organic base such as ethanolamine,
10 diethanolamine, triethanolamine, tromethamine, N-methylglucamine, and the like.

"PK" refers to receptor protein tyrosine kinase (RTKs), non-receptor or "cellular" tyrosine kinase (CTKs) and serine-threonine kinases (STKs).

"Modulation" or "modulating" refers to the alteration of the catalytic activity of protein kinases, in particular CDK2 kinase. In particular, modulating refers to the
15 activation of the catalytic activity, preferably the activation or inhibition of the catalytic activity of, protein kinases, in particular CDK2 kinase, depending on the concentration of the compound or salt to which the protein kinases, in particular CDK2 kinase, is exposed or, more preferably, the inhibition of the catalytic activity of protein kinases, in particular CDK2 kinase.

20 "Therapeutically effective amount" refers to that amount of the compound being administered which will relieve to some extent one or more of the symptoms of the disorder being treated. In reference to the treatment of cancer, a therapeutically effective amount refers to that amount which has the effect of:

- (1) reducing the size of the tumor;
- 25 (2) inhibiting (that is, slowing to some extent, preferably stopping) tumor metastasis;
- (3) inhibiting to some extent (that is, slowing to some extent, preferably stopping) tumor growth, and/or,
- (4) relieving to some extent (or, preferably, eliminating) one or more
30 symptoms associated with the cancer.

The term "function" refers to the cellular role of a protein kinase. The

protein kinase family includes members that regulate many steps in signaling cascades, including cascades controlling cell growth, migration, differentiation, gene expression, muscle contraction, glucose metabolism, cellular protein synthesis, and regulation of the cell cycle.

5 The term "catalytic activity," in the context of the invention, defines the rate at which a protein kinase phosphorylates a substrate. Catalytic activity can be measured, for example, by determining the amount of a substrate converted to a product as a function of time. Phosphorylation of a substrate occurs at the active-site of a protein kinase. The active-site is normally a cavity in which the substrate
10 binds to the protein kinase and is phosphorylated.

 The term "substrate" as used herein refers to a molecule phosphorylated by a protein kinase. The substrate is preferably a peptide and more preferably a protein.

 The term "activates" refers to increasing the cellular function of a protein kinase. The protein kinase function is preferably the interaction with a natural
15 binding partner and most preferably catalytic activity.

 The term "inhibit" refers to decreasing the cellular function of a protein kinase. The protein kinase function is preferably the interaction with a natural binding partner and most preferably catalytic activity.

 The term "modulates" refers to altering the function of a protein kinase by
20 increasing or decreasing the probability that a complex forms between a protein kinase and a binding partner. A modulator preferably increases the probability that such a complex forms between the protein kinase and the binding partner, more preferably increases or decreases the probability that a complex forms between the protein kinase and the binding partner depending on the concentration of the
25 compound exposed to the protein kinase, and most preferably decreases the probability that a complex forms between the protein kinase and the binding partner.

 A modulator preferably activates the catalytic activity of a protein kinase, more preferably activates or inhibits the catalytic activity of a protein kinase depending on the concentration of the compound exposed to the protein kinase, or most preferably
30 inhibits the catalytic activity of a protein kinase.

 The term "complex" refers to an assembly of at least two molecules bound to

one another. Signal transduction complexes often contain at least two protein molecules bound to one another.

The term "binding partner" refers to a compound that binds to a protein kinase in cells. Binding partners can play a role in propagating a signal in a protein kinase signal transduction process. A change in the interaction between a protein kinase and a binding partner can manifest itself as an increased or decreased probability that the interaction forms, or an increased or decreased concentration of the protein kinase/binding partner complex. A binding partner may be a natural binding partners, in which case the binding partner is one which binds to the protein kinase during a cell's normal function. However, other molecules which bind to the protein kinase are also the protein kinase's binding partners.

A protein kinase binding partner can bind to a protein kinase's intracellular region with high affinity. High affinity represents an equilibrium binding constant on the order of 10^{-6} M or less. In addition, a natural binding partner can also transiently interact with a protein kinase intracellular region and chemically modify it. Protein kinase natural binding partners are chosen from a group that includes, but is not limited to, SRC homology 2 (SH2) or 3 (SH3) domains, other phosphoryl tyrosine binding (PTB) domains, guanine nucleotide exchange factors, protein phosphatases, and other protein kinases. Methods of determining changes in interactions between protein kinases and their natural binding partners are readily available in the art.

The term "contacting" as used herein refers to mixing a solution comprising a compound of the invention with a liquid medium bathing the cells of the methods.

The solution comprising the compound may also comprise another component, such as dimethylsulfoxide (DMSO), which facilitates the uptake of the compound or compounds into the cells of the methods. The solution comprising the compound of the invention may be added to the medium bathing the cells by utilizing a delivery apparatus, such as a pipet-based device or syringe-based device.

The compounds of the invention preferably modulate the activity of the protein kinase *in vitro*. These compounds preferably show positive results in one or more *in vitro* assays for an activity corresponding to treatment of the disease or

disorder in question (such as the assays described in the Examples below).

The invention also features a method of identifying compounds that modulate the function of protein kinase, comprising the following steps: (a) contacting cells expressing the protein kinase with the compound; and (b) monitoring an effect upon the cells. The effect upon the cells is preferably a change or an absence of a change in cell phenotype, more preferably it is a change or an absence of a change in cell proliferation, even more preferably it is a change or absence of a change in the catalytic activity of the protein kinase, and most preferably it is a change or absence of a change in the interaction between the protein kinase with a natural binding partner, as described herein.

The term "monitoring" refers to observing the effect of adding the compound to the cells of the method. The effect can be manifested in a change in cell phenotype, cell proliferation, protein kinase catalytic activity, or in the interaction between a protein kinase and a natural binding partner.

The term "effect" describes a change or an absence of a change in cell phenotype or cell proliferation. "Effect" can also describe a change or an absence of a change in the catalytic activity of the protein kinase. "Effect" can also describe a change or an absence of a change in an interaction between the protein kinase and a natural binding partner.

The term "cell phenotype" refers to the outward appearance of a cell or tissue or the function of the cell or tissue. Examples of cell phenotype are cell size (reduction or enlargement), cell proliferation (increased or decreased numbers of cells), cell differentiation (a change or absence of a change in cell shape), cell survival, apoptosis (cell death), or the utilization of a metabolic nutrient (*e.g.*, glucose uptake). Changes or the absence of changes in cell phenotype are readily measured by techniques known in the art.

In a preferred embodiment, the invention features a method for identifying the compounds of the invention, comprising the following steps: (a) lysing the cells to render a lysate comprising protein kinase; (b) adsorbing the protein kinase to an antibody; (c) incubating the adsorbed protein kinase with a substrate or substrates; and (d) adsorbing the substrate or substrates to a solid support or antibody.

In other preferred embodiments, the step of monitoring the effect on the cells comprises measuring the phosphate concentration of the substrate or substrates.

The term "antibody" refers to an antibody (e.g., a monoclonal or polyclonal antibody), or antibody fragment, having specific binding affinity to protein kinase or
5 its fragment.

By "specific binding affinity" is meant that the antibody binds to target (protein kinase) polypeptides with greater affinity than it binds to other polypeptides under specified conditions. Antibodies having specific binding affinity to a protein kinase may be used in methods for detecting the presence and/or amount of a protein
10 kinase in a sample by contacting the sample with the antibody under conditions such that an immunocomplex forms and detecting the presence and/or amount of the antibody conjugated to the protein kinase. Diagnostic kits for performing such methods may be constructed to include a first container containing the antibody and a second container having a conjugate of a binding partner of the antibody and a
15 label, such as, for example, a radioisotope. The diagnostic kit may also include notification of an FDA approved use and instructions therefor.

The term "polyclonal" refers to antibodies that are heterogenous populations of antibody molecules derived from the sera of animals immunized with an antigen or an antigenic functional derivative thereof. For the production of polyclonal
20 antibodies, various host animals may be immunized by injection with the antigen. Various adjuvants may be used to increase the immunological response, depending on the host species.

"Monoclonal antibodies" are substantially homogenous populations of antibodies to a particular antigen. They may be obtained by any technique which
25 provides for the production of antibody molecules by continuous cell lines in culture. Monoclonal antibodies may be obtained by methods known to those skilled in the art. See, for example, Kohler, et al., *Nature* 256:495-497 (1975), and U.S. Patent No. 4,376,110.

The term "antibody fragment" refers to a portion of an antibody, often the
30 hypervariable region and portions of the surrounding heavy and light chains, that displays specific binding affinity for a particular molecule. A hypervariable region

is a portion of an antibody that physically binds to the polypeptide target.

The invention also features a method of regulating kinase signal transduction comprising administering to a subject a therapeutically effective amount of a compound of the invention as described herein.

5 Furthermore, the invention features a method of preventing or treating an abnormal condition in an organism, where the abnormal condition is associated with an aberration in a signal transduction pathway characterized by an interaction between a protein kinase and a natural binding partner, where the method comprises the following steps: (a) administering a compound of the invention as described
10 herein; and (b) promoting or disrupting the abnormal interaction. The organism is preferably a mammal and the abnormal condition is preferably cancer. In other preferred embodiments, the abnormal condition is an angiogenesis-related disorder or a dermatologic, ophthalmic, neurologic, cardiovascular, or immune disorder. Some specific abnormal conditions include hypertension, depression, generalized
15 anxiety disorder, phobias, post-traumatic stress syndrome, avoidant personality disorder, sexual dysfunction, eating disorders, obesity, chemical dependencies, cluster headache, migraine, pain, Alzheimer's disease, obsessive-compulsive disorder, panic disorder, memory disorders, Parkinson's disease, endocrine disorders, vasospasm, cerebellar ataxia, and gastrointestinal tract disorders.

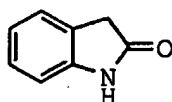
20 The term "aberration," in conjunction with a signal transduction process, refers to a protein kinase that is over- or under-expressed in an organism, mutated such that its catalytic activity is lower or higher than wild-type protein kinase activity, mutated such that it can no longer interact with a natural binding partner, is no longer modified by another protein kinase or protein phosphatase, or no longer
25 interacts with a natural binding partner.

 The term "promoting or disrupting the abnormal interaction" refers to a method that can be accomplished by administering a compound of the invention to cells or tissues in an organism. A compound can promote an interaction between a protein kinase and natural binding partners by forming favorable interactions with
30 multiple atoms at the complex interface. Alternatively, a compound can inhibit an interaction between a protein kinase and natural binding partners by compromising

favorable interactions formed between atoms at the complex interface.

The term "indolinone" is used as that term is commonly understood in the art and includes a large subclass of substituted or unsubstituted compounds that are capable of being synthesized from an aldehyde moiety and an oxindole moiety.

- 5 The term "oxindole" refers to an oxindole compound substituted with chemical substituents. Oxindole compounds are of the general structure:



PREFERRED EMBODIMENTS

- 10 In preferred embodiments, the invention relates to a compound of Formula (I) where

- (a) each R_1 is independently and optionally selected from the group consisting of:
- (i) hydrogen;
- 15 (ii) saturated alkyl comprising a branched or straight chain of one to five carbon atoms;
- (iii) an aromatic or heteroaromatic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, and ester; and
- 20 (iv) an aliphatic or heteroaliphatic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, ester, and an aromatic or heteroaromatic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen,
- 25 trihalomethyl, carboxylate, amino, nitro, and ester;
- (v) an alcohol of formula $-(X_1)_{n1}-OH$ or an alkoxyalkyl of formula $-(X_2)_{n2}-O-X_3$, where

- X_1 , X_2 , and X_3 are independently selected from the group consisting of alkyl comprising a branched or straight chain of one to five carbon atoms and a six-
- 30 membered aromatic ring; and $n1$ and $n2$ are independently 0 or 1;

- (vi) a halogen;
- (vii) a carboxylic acid of formula $-(X_4)_{n4}-COOH$ or ester of formula $-(X_5)_{n5}-COO-X_6$, where X_4 , X_5 , and X_6 are independently of alkyl comprising a branched or straight chain of one to five carbon atoms, and $n4$ and $n5$ are independently 0 or 1;
- 5 (viii) an amide of formula $-(X_7)_{n7}-NHCOX_8$, or of formula $-(X_9)_{n9}-CONX_{10}X_{11}$, where
 X_7 and X_9 are each independently alkyl comprising a branched or straight chain of one to five carbon atoms, $n7$ and $n9$ are independently 0 or 1; X_8 , X_{10} , and X_{11} are each independently hydrogen or alkyl comprising a branched or straight chain of one
10 to five carbon atoms; and where X_{10} and X_{11} taken together form a five-membered or six-membered aliphatic or heteroaliphatic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;
- (ix) a sulfonamide of formula $-(X_{12})_{n12}-SO_2NX_{13}X_{14}$, wherein X_{12} is alkyl
15 comprising a branched or straight chain of one to five carbon atoms; $n12$ is 0 or 1; X_{13} , and X_{14} are independently selected from the group consisting of hydrogen, alkyl, and five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where the alkyl and each ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl,
20 halogen, hydroxy, trihalomethyl, carboxylate, amino, nitro, and ester; or
where X_{13} and X_{14} taken together form a five-membered or six-membered aliphatic or heteroaliphatic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;
- 25 (x) a sulfone of formula $-(X_{15})_{n15}-SO_3H$, where X_{15} is alkyl comprising a branched or straight chain of one to five carbon atoms; and $n15$ is 0, 1, or 2; and
- (xi) an amine of formula $-(X_{16})_{n16}-NX_{17}X_{18}$, where X_{16} is alkyl comprising a branched or straight chain of one to five carbon atoms; $n16$ is 0 or 1, and X_{17} and X_{18} are independently selected from the group consisting of hydrogen, saturated or
30 unsaturated alkyl, and five-membered or six-membered aromatic, heteroaromatic, or aliphatic ring;

- (xii) a ketone of formula $-(X_{19})_{n19}-CO-X_{20}$, where X_{19} and X_{20} are independently selected from the group consisting of alkyl and five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where the alkyl and each ring is optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring, halogen, trihalomethyl, carboxylate, amino, nitro, and ester; and where, and $n19$ is 0 or 1;
- (xiii) a nitro group of the formula $-NO_2$; or
- (xiv) two adjacent R_1 groups together along with two adjacent A, B, D, or E form a five-membered or six-membered aliphatic or heteroaliphatic ring;
- (b) R_2 , R_3 , and R_4 are hydrogen;
- (c) A, B, D, and E are each independently carbon or nitrogen, provided that none, one, or two of A, B, D, and E is nitrogen, preferably A, B, D, and E are carbon; and
- (d) provided that when any of A, B, D, or E is nitrogen, no R_1 is attached to the A, B, D, or E;
- (d) G is nitrogen or oxygen, with the proviso that when G is oxygen, R_5 is absent;
- (e) m is 2, 3, or 4; and
- (f) q is 1, 2, or 3; or
- a pharmaceutically acceptable salt thereof.

More preferably, in the compounds of Formula I (a) each R_1 is independently and optionally selected from the group consisting of (i) hydrogen; (ii) methyl, ethyl, propyl, ethylene, propylene, or butylene; (iii) an alcohol of formula $-(X_1)_{n1}-OH$ or an alkoxyalkyl of formula $-(X_2)_{n2}-O-X_3$, wherein X_1 , X_2 , and X_3 are independently selected from the group consisting of methylene, ethylene, or propylene, and $n1$ and $n2$ are independently 0 or 1; (iv) fluoro, chloro, or bromo; (v) a carboxylic acid of formula $-(X_4)_{n4}-COOH$, wherein X_4 is selected from the group consisting of methylene, ethylene, or propylene, and $n4$ is 0 or 1; (vi) an amide of formula $-(X_7)_{n7}-NHCOX_8$, or of formula $-(X_9)_{n9}-CONX_{10}X_{11}$, where X_7 and X_9 are each independently selected from the group consisting of methylene, ethylene, and

- propylene, and n_7 and n_9 are independently 0 or 1, X_8 , X_{10} , and X_{11} are each independently selected from the group consisting of hydrogen, methyl, ethyl, and propyl, or where X_{10} and X_{11} taken together form a five-membered or six-membered heteroaliphatic ring; (vii) a sulfonamide of formula $-(X_{12})_{n_{12}}-SO_2NX_{13}X_{14}$, where X_{12} is selected from the group consisting of methylene, ethylene, or propylene, and n_{12} is 0 or 1, X_{13} , and X_{14} are independently selected from the group consisting of hydrogen, methyl, ethyl, propyl, and phenyl, the methyl, ethyl, propyl, and phenyl are optionally substituted with one or more substituents independently selected from the group consisting of halogen, hydroxy, carboxylate, amino, nitro, and ester, or where X_{13} and X_{14} taken together form a fused bicyclic ring; or (viii) two adjacent R_i groups together along with two adjacent A, B, D, or E form a six-membered heteroaliphatic ring; (b) A, B, D, and E are carbon; (c) X is nitrogen or oxygen; (d) m is 4; and (e) q is 2.

- Even more preferably, R_i in Formula I is independently selected from the group consisting of methyl, methoxy, 2-hydroxyethyl, phenyl, 2-methoxyphenyl, 3-methoxyphenyl, 4-methoxyphenyl, 4-fluorophenyl, 4-chlorophenyl, 4-bromophenyl, 4-iodophenyl, 3-carboxyphenyl, pyridyl, $-NHC(O)CH_3$, $-C(O)N(CH_3)_2$, $-COOH$, $-SO_2NH(CH_3)$, $-SO_2NH_2$, $-SO_2N(CH_3)_2$, $-SO_2NH(CH(CH_3)_2)$, $-SO_2NH(C_6H_5)$, $-SO_2NH(3\text{-chlorophenyl})$, and $-SO_2N(CH_3)(3\text{-chlorophenyl})$.

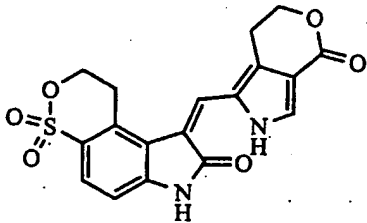
The preferred indolinone compounds of the invention are listed in Table 1.

Table 1.

Compound Number	Compound Name
IN-001	4-methyl-2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid methylamide
IN-002	2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid methylamide
IN-003	1-(4-methyl-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-004	1-(5-methoxy-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-005	1-(6-methoxy-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-006	1-(5-bromo-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-

Compound Number	Compound Name
	pyrano[3,4-c]pyrrol-4-one
IN-007	1-(5-chloro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-008	1-[4-(2-hydroxy-ethyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-009	1-(2-oxo-5-phenyl-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-010	2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid amide
IN-011	2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid dimethylamide
IN-012	2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid isopropylamide
IN-013	2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid phenylamide
IN-014	N-[2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indol-6-yl]-acetamide
IN-015	1-(2-oxo-6-phenyl-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-016	1-(5-fluoro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-017	2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indole-5-carboxylic acid
IN-018	2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indole-6-carboxylic acid
IN-019	1-(6-chloro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-020	1-(6-bromo-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-021	1-[6-(2-methoxy-phenyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-022	1-[6-(3-methoxy-phenyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-023	1-[6-(4-methoxy-phenyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-024	1-[6-(4-fluoro-phenyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-025	1-(2-oxo-6-pyridin-3-yl-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-026	1-[5-(2,3-dihydro-indole-1-sulfonyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-027	1-[5-(3,4-dihydro-2H-quinoline-1-sulfonyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-028	1-[5-(3,4-dihydro-1H-isoquinoline-2-sulfonyl)-2-oxo-1,2-dihydro-indol-

Compound Number	Compound Name
	3-ylidenemethyl]-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-029	1-[5-(5-bromo-2,3-dihydro-indole-1-sulfonyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-030	2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid (3-chloro-phenyl)-methyl-amide
IN-031	1-(6-fluoro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-032	1-(5-chloro-4-methyl-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-033	1-(7-chloro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-034	3-[2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indol-5-yl]-benzoic acid
IN-035	3-[2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indol-6-yl]-benzoic acid
IN-036	2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid (3-chloro-phenyl)-amide
IN-037	1-(5-bromo-4-methyl-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-038	1-(2-oxo-5-phenyl-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one
IN-039	1-(2-oxo-6-phenyl-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one
IN-040	1-(6-bromo-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one
IN-041	1-(6-chloro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one
IN-042	2-oxo-3-(4-oxo-4,5,6,7-tetrahydro-2H-pyrrolo[3,4-c]pyridin-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid dimethylamide
IN-043	2-oxo-3-(4-oxo-4,5,6,7-tetrahydro-2H-pyrrolo[3,4-c]pyridin-1-ylmethylene)-2,3-dihydro-1H-indole-5-carboxylic acid dimethylamide
IN-044	1-[2-oxo-5-(pyrrolidine-1-carbonyl)-1,2-dihydro-indol-3-ylidenemethyl]-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one
IN-045	1-[5-(morpholine-4-carbonyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one
IN-046	1-[4-(2-hydroxy-ethyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one
IN-047	1-(5-methoxy-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one
IN-048	1-(5-fluoro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one
IN-049	1-(5-bromo-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one

Compound Number	Compound Name
IN-050	2-oxo-3-(4-oxo-4,5,6,7-tetrahydro-2H-pyrrolo[3,4-c]pyridin-1-ylmethylene)-2,3-dihydro-1H-indole-5-carboxylic acid
IN-051	2-oxo-3-(4-oxo-4,5,6,7-tetrahydro-2H-pyrrolo[3,4-c]pyridin-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid amide
IN-052	2-oxo-3-(4-oxo-4,5,6,7-tetrahydro-2H-pyrrolo[3,4-c]pyridin-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid methylamide
IN-053	
IN-054	1-[4-(2-hydroxyethyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-5-methyl-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one
IN-055	1-(5-bromo-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-5-methyl-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one
IN-056	3-(5-methyl-4-oxo-4,5,6,7-tetrahydro-2H-pyrrolo[3,4-c]pyridin-1-ylmethylene)-2-oxo-2,3-dihydro-1H-indole-5-carboxylic acid
IN-057	3-(5-methyl-4-oxo-4,5,6,7-tetrahydro-2H-pyrrolo[3,4-c]pyridin-1-ylmethylene)-2-oxo-2,3-dihydro-1H-indole-5-sulfonic acid methylamide
IN-058	3-(5-methyl-4-oxo-4,5,6,7-tetrahydro-2H-pyrrolo[3,4-c]pyridin-1-ylmethylene)-2-oxo-2,3-dihydro-1H-indole-5-sulfonic acid dimethylamide
IN-059	1-(2-oxo-1,2-dihydro-pyrrolo[2,3-b]pyridin-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one
IN-060	4-(3-chloro-4-fluoro-phenylamino)-5-(4-oxo-2,4,6,7-tetrahydropyrano[3,4-c]-pyrrol-1-ylmethylene)-5,7-dihydro-pyrrolo[2,3-d]pyrimidin-6-one

Some of the above compounds have the structure of Formula II, with the substituents as defined in Table 2.

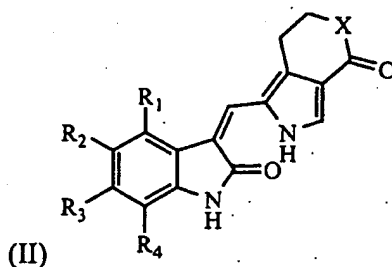
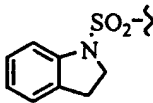
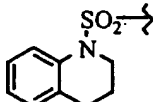
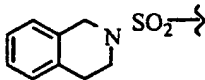
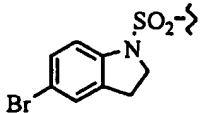
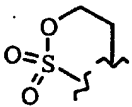
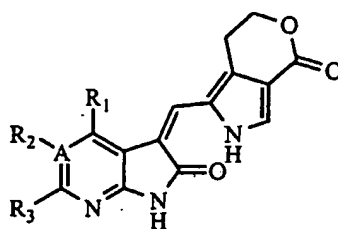


Table 2

Compound Number	R ₁	R ₂	R ₃	R ₄	X
IN-001	-CH ₃	-SO ₂ NHCH ₃	H	H	O
IN-002	H	-SO ₂ NHCH ₃	H	H	O
IN-003	-CH ₃	H	H	H	O
IN-004	H	-OCH ₃	H	H	O
IN-005	H	H	-OCH ₃	H	O
IN-006	H	Br	H	H	O
IN-007	H	Cl	H	H	O
IN-008	-CH ₂ CH ₂ OH	H	H	H	O
IN-009	H	phenyl	H	H	O
IN-010	H	-SO ₂ NH ₂	H	H	O
IN-011	H	-SO ₂ N(CH ₃) ₂	H	H	O
IN-012	H	-SO ₂ NHCH(CH ₃) ₂	H	H	O
IN-013	H	-SO ₂ NH(phenyl)	H	H	O
IN-014	H	H	-NHC(O)CH ₃	H	O
IN-015	H	H	phenyl	H	O
IN-016	H	F	H	H	O
IN-017	H	-COOH	H	H	O
IN-018	H	H	-COOH	H	O
IN-019	H	H	Cl	H	O
IN-020	H	H	Br	H	O
IN-021	H	H	2-methoxyphenyl	H	O
IN-022	H	H	3-methoxyphenyl	H	O
IN-023	H	H	4-methoxyphenyl	H	O
IN-024	H	H	4-fluorophenyl	H	O
IN-025	H	H	3-pyridyl	H	O
IN-026	H		H	H	O
IN-027	H		H	H	O
IN-028	H		H	H	O

Compound Number	R ₁	R ₂	R ₃	R ₄	X
IN-029	H		H	H	O
IN-030	H	-SO ₂ NCH ₃ (3-chlorophenyl)	H	H	O
IN-031	H	H	F	H	O
IN-032	-CH ₃	Cl	H	H	O
IN-033	H	H	H	Cl	O
IN-034	H	3-carboxyphenyl	H	H	O
IN-035	H	H	3-carboxyphenyl	H	O
IN-036	H	-SO ₂ NH(3-chlorophenyl)	H	H	O
IN-037	-CH ₃	Br	H	H	O
IN-038	H	phenyl	H	H	NH
IN-039	H	H	phenyl	H	NH
IN-040	H	H	Br	H	NH
IN-041	H	H	Cl	H	NH
IN-042	H	-SO ₂ N(CH ₃) ₂	H	H	NH
IN-043	H	-C(O)N(CH ₃) ₂	H	H	NH
IN-044	H	-C(O)(pyrrol-1-yl)	H	H	NH
IN-045	H	-C(O)(morpholin-4-yl)	H	H	NH
IN-046	-CH ₂ CH ₂ OH	H	H	H	NH
IN-047	H	-OCH ₃	H	H	NH
IN-048	H	F	H	H	NH
IN-049	H	Br	H	H	NH
IN-050	H	-COOH	H	H	NH
IN-051	H	-SO ₂ NH ₂	H	H	NH
IN-052	H	-SO ₂ NHCH ₃	H	H	NH
IN-053	taken together form 		H	H	NH
IN-054	H		H	H	NMe
IN-055	H	Br	H	H	NMe
IN-056	H	-COOH	H	H	NMe
IN-057	H	-SO ₂ NHCH ₃	H	H	NMe
IN-058	H	-SO ₂ NHCH ₃	H	H	NMe

Two of the compounds of Table 1 have the structure of Formula III, with the substituents as defined in Table 3.



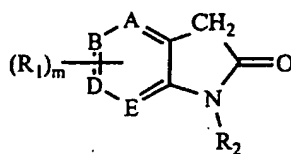
(III)

Table 3

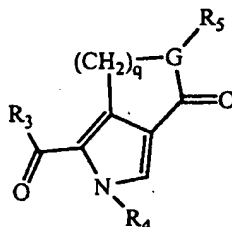
Compound Number	R ₁	R ₂	R ₃	A
IN-059	H	H	H	C
IN-060	3-chloro-4-fluoro-phenylamino	Does not exist	H	N

GENERAL SYNTHESIS

In general, the compounds of Formula (I) can be prepared by reacting an oxindole having the following structure



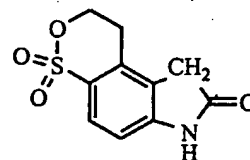
with an aldehyde or ketone having the following structure



wherein R₁, R₂, R₃, R₄, R₅, A, B, D, E, G, m, and q are as described in the Summary of the Invention.

The oxindole selected from the group consisting of 4-methyl-2-oxo-2,3-

- dihydro-1H-indole-5-sulfonic acid methylamide, 2-oxo-2,3-dihydro-1H-indole-5-sulfonic acid methylamide, 4-methyl-2-oxo-1,2-dihydro-indole, 5-methoxy-2-oxo-1,2-dihydro-indole, 6-methoxy-2-oxo-1,2-dihydro-indole, 5-bromo-2-oxo-1,2-dihydro-indole, 5-chloro-2-oxo-1,2-dihydro-indole, 4-(2-hydroxy-ethyl)-2-oxo-1,2-dihydro-indole, 2-oxo-5-phenyl-1,2-dihydro-indole, 2-oxo-2,3-dihydro-1H-indole-5-sulfonic acid amide, 2-oxo-2,3-dihydro-1H-indole-5-sulfonic acid dimethylamide, 2-oxo-2,3-dihydro-1H-indole-5-sulfonic acid isopropylamide, 2-oxo-2,3-dihydro-1H-indole-5-sulfonic acid phenylamide, N-(2-oxo-2,3-dihydro-1H-indol-6-yl)-acetamide, 2-oxo-6-phenyl-1,2-dihydro-indole, 5-fluoro-2-oxo-1,2-dihydro-indole, 2-oxo-2,3-dihydro-1H-indole-5-carboxylic acid, 2-oxo-2,3-dihydro-1H-indole-6-carboxylic acid, 6-chloro-2-oxo-1,2-dihydro-indole, 6-bromo-2-oxo-1,2-dihydro-indole, 6-(2-methoxy-phenyl)-2-oxo-1,2-dihydro-indole, 6-(3-methoxy-phenyl)-2-oxo-1,2-dihydro-indole, 6-(4-methoxy-phenyl)-2-oxo-1,2-dihydro-indole, 6-(4-fluoro-phenyl)-2-oxo-1,2-dihydro-indole, 2-oxo-6-pyridin-3-yl-1,2-dihydro-indole, 5-(2,3-dihydro-indole-1-sulfonyl)-2-oxo-1,2-dihydro-indole, 5-(3,4-dihydro-2H-quinoline-1-sulfonyl)-2-oxo-1,2-dihydro-indole, 5-(3,4-dihydro-1H-isoquinoline-2-sulfonyl)-2-oxo-1,2-dihydro-indole, 5-(5-bromo-2,3-dihydro-indole-1-sulfonyl)-2-oxo-1,2-dihydro-indole, 2-oxo-2,3-dihydro-1H-indole-5-sulfonic acid (3-chloro-phenyl)-methyl-amide, 6-fluoro-2-oxo-1,2-dihydro-indole, 5-chloro-4-methyl-2-oxo-1,2-dihydro-indole, 7-chloro-2-oxo-1,2-dihydro-indole, 3-(2-oxo-2,3-dihydro-1H-indol-5-yl)-benzoic acid, 3-(2-oxo-2,3-dihydro-1H-indol-6-yl)-benzoic acid, 2-oxo-2,3-dihydro-1H-indole-5-sulfonic acid (3-chloro-phenyl)-amide, 5-bromo-4-methyl-2-oxo-1,2-dihydro-indole, 2-oxo-2,3-dihydro-1H-indole-5-carboxylic acid dimethylamide, 2-oxo-5-(pyrrolidine-1-carbonyl)-1,2-dihydro-indole, 5-



- (morpholine-4-carbonyl)-2-oxo-1,2-dihydro-indole, and

The aldehyde is selected from the group consisting of 4-oxo-2,4,6,7-tetrahydro-2-formylpyrano[3,4-c]pyrrole and 4-oxo-4,5,6,7-tetrahydro-(1H-2-formyl-pyrrolo)[3,4-c]pyridine.

The reaction is carried out in the presence of a base. The base is preferably a

nitrogen base or an inorganic base. "Nitrogen bases" are commonly used in the art and are selected from acyclic and cyclic amines. Examples of nitrogen bases include, but are not limited to, ammonia, methylamine, trimethylamine, triethylamine, aniline, 1,8-diazabicyclo[5.4.0]undec-7-ene, diisopropylethylamine, pyrrolidine, piperidine, and pyridine or substituted pyridine (*e.g.*, 2,6-di-
5 tertbutylpyridine). "Inorganic bases" are bases that do not contain any carbon atoms. Examples of inorganic bases include, but are not limited to, hydroxide, phosphate, bisulfate, hydrosulfide (SH⁻), and amide anions. Those skilled in the art know which nitrogen base or inorganic base would match the requirements of the
10 reaction conditions. In certain embodiments of the invention, the base used may be pyrrolidine or piperidine. In other embodiments the base may be the hydroxide anion, preferably used as its sodium or potassium salt.

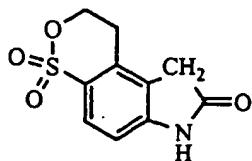
The synthesis of the compounds of the invention generally takes place in a solvent. The solvent of the reaction is preferably a protic solvent or an aprotic
15 solvent. "Protic solvents" are those that are capable of donating a proton to a solute. Examples of protic solvents include, but are not limited to, alcohols and water. "Aprotic solvents" are those solvents that, under normal reaction conditions, do not donate a proton to a solute. Typical organic solvents, such as hexane, toluene, benzene, methylene chloride, dimethylformamide, chloroform, tetrahydrofuran, are
20 some of the examples of aprotic solvents. Other aprotic solvents are also within the scope of the present invention. In some preferred embodiments, the solvent of the reaction is an alcohol, which may preferably be isopropanol or most preferably ethanol. Water is another preferred protic solvent. Dimethylformamide, known in the chemistry art as DMF, is a preferred aprotic solvent.

25 The synthetic method of the invention calls for the reaction to take place at elevated temperatures which are temperatures that are greater than room temperature. More preferably, the elevated temperature is preferably about 30-150 °C, more preferably is about 80-100 °C, and most preferably is about 80-90 °C, which is about the temperature at which ethanol boils (*i.e.*, the boiling point of
30 ethanol). By "about" a certain temperature it is meant that the temperature range is preferably within 10 °C of the listed temperature, more preferably within 5 °C of the

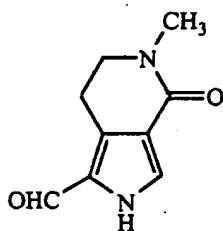
listed temperature, and most preferably within 2 °C of the listed temperature.

Therefore, by way of example, by "about 80 °C" it is meant that the temperature range is preferably 80±10 °C, more preferably 80±5 °C, and most preferably 80±2 °C.

- 5 The oxindole in the combinatorial library is preferably selected from the group consisting of 4-methyl-2-oxo-2,3-dihydro-1H-indole-5-sulfonic acid methylamide, 2-oxo-2,3-dihydro-1H-indole-5-sulfonic acid methylamide, 4-methyl-2-oxo-1,2-dihydro-indole, 5-methoxy-2-oxo-1,2-dihydro-indole, 6-methoxy-2-oxo-1,2-dihydro-indole, 5-bromo-2-oxo-1,2-dihydro-indole, 5-chloro-2-oxo-1,2-dihydro-10 indole, 4-(2-hydroxy-ethyl)-2-oxo-1,2-dihydro-indole, 2-oxo-5-phenyl-1,2-dihydro-indole, 2-oxo-2,3-dihydro-1H-indole-5-sulfonic acid amide, 2-oxo-2,3-dihydro-1H-indole-5-sulfonic acid dimethylamide, 2-oxo-2,3-dihydro-1H-indole-5-sulfonic acid isopropylamide, 2-oxo-2,3-dihydro-1H-indole-5-sulfonic acid phenylamide, N-(2-oxo-2,3-dihydro-1H-indol-6-yl)-acetamide, 2-oxo-6-phenyl-1,2-dihydro-indole, 5-15 fluoro-2-oxo-1,2-dihydro-indole, 2-oxo-2,3-dihydro-1H-indole-5-carboxylic acid, 2-oxo-2,3-dihydro-1H-indole-6-carboxylic acid, 6-chloro-2-oxo-1,2-dihydro-indole, 6-bromo-2-oxo-1,2-dihydro-indole, 6-(2-methoxy-phenyl)-2-oxo-1,2-dihydro-indole, 6-(3-methoxy-phenyl)-2-oxo-1,2-dihydro-indole, 6-(4-methoxy-phenyl)-2-oxo-1,2-dihydro-indole, 6-(4-fluoro-phenyl)-2-oxo-1,2-dihydro-indole, 2-oxo-6-pyridin-3-yl-20 1,2-dihydro-indole, 5-(2,3-dihydro-indole-1-sulfonyl)-2-oxo-1,2-dihydro-indole, 5-(3,4-dihydro-2H-quinoline-1-sulfonyl)-2-oxo-1,2-dihydro-indole, 5-(3,4-dihydro-1H-isoquinoline-2-sulfonyl)-2-oxo-1,2-dihydro-indole, 5-(5-bromo-2,3-dihydro-indole-1-sulfonyl)-2-oxo-1,2-dihydro-indole, 2-oxo-2,3-dihydro-1H-indole-5-sulfonic acid (3-chloro-phenyl)-methyl-amide, 6-fluoro-2-oxo-1,2-dihydro-indole, 5-25 chloro-4-methyl-2-oxo-1,2-dihydro-indole, 7-chloro-2-oxo-1,2-dihydro-indole, 3-(2-oxo-2,3-dihydro-1H-indol-5-yl)-benzoic acid, 3-(2-oxo-2,3-dihydro-1H-indol-6-yl)-benzoic acid, 2-oxo-2,3-dihydro-1H-indole-5-sulfonic acid (3-chloro-phenyl)-amide, 5-bromo-4-methyl-2-oxo-1,2-dihydro-indole, 2-oxo-2,3-dihydro-1H-indole-5-carboxylic acid dimethylamide, 2-oxo-5-(pyrrolidine-1-carbonyl)-1,2-dihydro-30 indole, 5-(morpholine-4-carbonyl)-2-oxo-1,2-dihydro-indole, and



The aldehyde is preferably selected from the group consisting of 4-oxo-2,4,6,7-tetrahydro-2-formylpyrano[3,4-c]pyrrole, 4-oxo-4,5,6,7-tetrahydro-(1H-2-



formyl-pyrrolo)[3,4-c]pyridine, and

5

The synthetic method of the invention may be accompanied by the step of screening a library for a compound of the desired activity and structure - thus, providing a method of synthesis of a compound by first screening for a compound having the desired properties and then chemically synthesizing that compound.

10

Utility

The present invention relates to compounds capable of regulating and/or modulating cellular signal transduction and, in preferred embodiments, receptor and non-receptor tyrosine kinase signal transduction.

Receptor kinase mediated signal transduction is initiated by extracellular
15 interaction with a specific growth factor (ligand), followed by receptor dimerization, transient stimulation of the intrinsic protein kinase activity and phosphorylation. Binding sites are thereby created for intracellular signal transduction molecules and lead to the formation of complexes with a spectrum of cytoplasmic signaling molecules that facilitate the appropriate cellular response (*e.g.*, cell division,
20 metabolic effects to the extracellular microenvironment). *See*, Schlessinger and Ullrich, 1992, *Neuron* 9:303-391.

It has been shown that tyrosine phosphorylation sites in growth factor receptors function as high-affinity binding sites for SH2 (*src* homology) domains of

signaling molecules. Fantl *et al.*, 1992, *Cell* 69:413-423; Songyang *et al.*, 1994, *Mol. Cell. Biol.* 14:2777-2785; Songyang *et al.*, 1993, *Cell* 72:767-778; and Koch *et al.*, 1991, *Science* 252:668-678. Several intracellular substrate proteins that associate with receptor kinases have been identified. They may be divided into two principal groups: (1) substrates which have a catalytic domain; and (2) substrates which lack such domain but serve as adapters and associate with catalytically active molecules. Songyang *et al.*, 1993, *Cell* 72:767-778. The specificity of the interactions between receptors and SH2 domains of their substrates is determined by the amino acid residues immediately surrounding the phosphorylated tyrosine residue. Differences in the binding affinities between SH2 domains and the amino acid sequences surrounding the phosphotyrosine residues on particular receptors are consistent with the observed differences in their substrate phosphorylation profiles. Songyang *et al.*, 1993, *Cell* 72:767-778. These observations suggest that the function of each receptor kinase is determined not only by its pattern of expression and ligand availability but also by the array of downstream signal transduction pathways that are activated by a particular receptor. Thus, phosphorylation provides an important regulatory step which determines the selectivity of signaling pathways recruited by specific growth factor receptors, as well as differentiation factor receptors.

Kinase signal transduction results in, among other responses, cell proliferation, differentiation and metabolism. Abnormal cell proliferation may result in a wide array of disorders and diseases, including the development of neoplasia such as carcinoma, sarcoma, leukemia, glioblastoma, hemangioma, psoriasis, arteriosclerosis, arthritis and diabetic retinopathy (or other disorders related to uncontrolled angiogenesis and/or vasculogenesis).

Target Diseases to be Treated by the Compounds of the Invention:

This invention is therefore directed to compounds which regulate, modulate and/or inhibit kinase signal transduction by affecting the enzymatic activity of the RKs and/or the non-receptor kinases and interfering with the signal transduced by such proteins. More particularly, the present invention is directed to compounds which regulate, modulate and/or inhibit the receptor tyrosine kinase (RTK) and/or

non-receptor kinase mediated signal transduction pathways as a therapeutic approach to cure many kinds of tumors, including but not limited to carcinoma, sarcoma, erythroblastoma, glioblastoma, meningioma, astrocytoma, melanoma and myoblastoma. Indications may include, but are not limited to brain cancers, bladder
5 cancers, ovarian cancers, gastric cancers, pancreas cancers, colon cancers, blood cancers, lung cancers and bone cancers.

The compounds described herein are useful for treating disorders related to unregulated kinase signal transduction, including cell proliferative disorders, fibrotic disorders and metabolic disorders.

10 Cell proliferative disorders which can be treated or further studied by the present invention include cancers, blood vessel proliferative disorders and mesangial cell proliferative disorders.

Blood vessel proliferative disorders refer to angiogenic and vasculogenic disorders generally resulting in abnormal proliferation of blood vessels. The
15 formation and spreading of blood vessels, or vasculogenesis and angiogenesis, respectively, play important roles in a variety of physiological processes such as embryonic development, corpus luteum formation, wound healing and organ regeneration. They also play a pivotal role in cancer development. Other examples of blood vessel proliferation disorders include arthritis, where new capillary blood
20 vessels invade the joint and destroy cartilage, and ocular diseases, like diabetic retinopathy, where new capillaries in the retina invade the vitreous, bleed and cause blindness. Conversely, disorders related to the shrinkage, contraction or closing of blood vessels, such as restenosis, are also implicated.

Fibrotic disorders refer to the abnormal formation of extracellular matrix.
25 Examples of fibrotic disorders include hepatic cirrhosis and mesangial cell proliferative disorders. Hepatic cirrhosis is characterized by the increase in extracellular matrix constituents resulting in the formation of a hepatic scar. Hepatic cirrhosis can cause diseases such as cirrhosis of the liver. An increased extracellular matrix resulting in a hepatic scar can also be caused by viral infection such as
30 hepatitis. Lipocytes appear to play a major role in hepatic cirrhosis. Other fibrotic disorders implicated include atherosclerosis (*see, below*).

Mesangial cell proliferative disorders refer to disorders brought about by abnormal proliferation of mesangial cells. Mesangial proliferative disorders include various human renal diseases, such as glomerulonephritis, diabetic nephropathy, malignant nephrosclerosis, thrombotic microangiopathy syndromes, transplant rejection, and glomerulopathies. The PDGF-R has been implicated in the maintenance of mesangial cell proliferation. Floege *et al.*, 1993, *Kidney International* 43:47S-54S.

PTKs have been associated with such cell proliferative disorders. For example, some members of the RTK family have been associated with the development of cancer. Some of these receptors, like the EGFR (Tuzi *et al.*, 1991, *Br. J. Cancer* 63:227-233; Torp *et al.*, 1992, *APMIS* 100:713-719) HER2/neu (Slamon *et al.*, 1989, *Science* 244:707-712) and the PDGF-R (Kumabe *et al.*, 1992, *Oncogene* 7:627-633) are overexpressed in many tumors and/or persistently activated by autocrine loops. In fact, in the most common and severe cancers these receptor overexpressions (Akbasak and Suner-Akbasak *et al.*, 1992, *J. Neurol. Sci.* 111:119-133; Dickson *et al.*, 1992, *Cancer Treatment Res.* 61:249-273; Korc *et al.*, 1992, *J. Clin. Invest.* 90:1352-1360) and autocrine loops (Lee and Donoghue, 1992, *J. Cell. Biol.* 118:1057-1070; Korc *et al.*, *supra*; Akbasak and Suner-Akbasak *et al.*, *supra*) have been demonstrated. For example, the EGFR receptor has been associated with squamous cell carcinoma, astrocytoma, glioblastoma, head and neck cancer, lung cancer and bladder cancer. HER2 has been associated with breast, ovarian, gastric, lung, pancreas and bladder cancer. The PDGF-R has been associated with glioblastoma, lung, ovarian, melanoma and prostate cancer. The RTK c-met has been generally associated with hepatocarcinogenesis and thus hepatocellular carcinoma. Additionally, c-met has been linked to malignant tumor formation. More specifically, the RTK c-met has been associated with, among other cancers, colorectal, thyroid, pancreatic and gastric carcinoma, leukemia and lymphoma. Additionally, over-expression of the c-met gene has been detected in patients with Hodgkin's disease, Burkitt's disease, and the lymphoma cell line.

The IGF-IR, in addition to being implicated in nutritional support and in type-II diabetes, has also been associated with several types of cancers. For

example, IGF-I has been implicated as an autocrine growth stimulator for several tumor types, e.g., human breast cancer carcinoma cells (Arteaga *et al.*, 1989, *J. Clin. Invest.* 84:1418-1423) and small lung tumor cells (Macauley *et al.*, 1990, *Cancer Res.* 50:2511-2517). In addition, IGF-I, integrally involved in the normal growth and differentiation of the nervous system, appears to be an autocrine stimulator of human gliomas. Sandberg-Nordqvist *et al.*, 1993, *Cancer Res.* 53:2475-2478. The importance of the IGF-IR and its ligands in cell proliferation is further supported by the fact that many cell types in culture (fibroblasts, epithelial cells, smooth muscle cells, T-lymphocytes, myeloid cells, chondrocytes, osteoblasts, the stem cells of the bone marrow) are stimulated to grow by IGF-I. Goldring and Goldring, 1991, *Eukaryotic Gene Expression* 1:301-326. In a series of publications, Baserga even suggests that IGF-I-R plays a central role in the mechanisms of transformation and, as such, could be a preferred target for therapeutic interventions for a broad spectrum of human malignancies. Baserga, 1995, *Cancer Res.* 55:249-252; Baserga, 1994, *Cell* 79:927-930; Coppola *et al.*, 1994, *Mol. Cell. Biol.* 14:4588-4595.

The association between abnormalities in RTKs and disease are not restricted to cancer, however. For example, RTKs have been associated with metabolic diseases like psoriasis, diabetes mellitus, wound healing, inflammation, and neurodegenerative diseases. These diseases include, but are not limited to hypertension, depression, generalized anxiety disorder, phobias, post-traumatic stress syndrome, avoidant personality disorder, sexual dysfunction, eating disorders, obesity, chemical dependencies, cluster headache, migraine, pain, Alzheimer's disease, obsessive-compulsive disorder, panic disorder, memory disorders, Parkinson's disease, endocrine disorders, vasospasm, cerebellar ataxia, and gastrointestinal tract disorders. For example, the EGF-R is indicated in corneal and dermal wound healing. Defects in the Insulin-R and the IGF-1R are indicated in type-II diabetes mellitus. A more complete correlation between specific RTKs and their therapeutic indications is set forth in Plowman *et al.*, 1994, *DN&P* 7:334-339.

Not only receptor type kinases, but also many cellular kinases (CKs) including src, abl, fps, yes, fyn, lyn, lck, blk, hck, fgr, yrk (reviewed by Bolen *et al.*, 1992, *FASEB J.* 6:3403-3409) are involved in the proliferative and metabolic signal

transduction pathway and thus in indications of the present invention. For example, mutated src (v-src) has been demonstrated as an oncoprotein (pp60^{v-src}) in chicken. Moreover, its cellular homologue, the proto-oncogene pp60^{c-src} transmits oncogenic signals of many receptors. For example, overexpression of EGF-R or HER2/neu in tumors leads to the constitutive activation of pp60^{c-src}, which is characteristic for the malignant cell but absent from the normal cell. On the other hand, mice deficient for the expression of c-src exhibit an osteopetrotic phenotype, indicating a key participation of c-src in osteoclast function and a possible involvement in related disorders. Similarly, Zap 70 is implicated in T-cell signaling.

Furthermore, the identification of CTK modulating compounds to augment or even synergize with RTK aimed blockers is an aspect of the present invention.

Finally, both RTKs and non-receptor type kinases have been connected to hyperimmune disorders.

The compounds of the present invention are also effective in treating diseases that are related to the PYK-2 protein. This protein, its cellular function, and diseases related to them are set forth in detail in U.S. Patent Number 5,837,524, issued November 17, 1998, and entitled "PYK2 RELATED PRODUCTS AND METHODS," and U.S. Patent Number 5,837,815, issued November 17, 1998, and entitled "PYK2 RELATED PRODUCTS AND METHODS," both of which are hereby incorporated by reference herein in their entirety, including any drawings.

In addition, the compounds of the present invention are effective against rheumatoid arthritis. Rheumatoid arthritis (RA) is a chronic inflammatory disease mediated by multiple cell types and cellular processes. Included in these are the infiltration of macrophages and T cells, and the involvement of angiogenesis. To investigate the utility of small molecule inhibitors for the treatment of RA, some of the compounds of the invention, which are tyrosine kinase inhibitors, were characterized in a rat collagen induced arthritis model. These compounds inhibit the tyrosine kinases FLK-1/KDR, PYK2, and ZAP-70 to varying degrees in biochemical kinase assays. (See the Examples below.) In addition, the compounds are active in cellular assays targeted to cells implicated in the pathogenesis of RA: inhibition of T cell proliferation mediated by ZAP-70 activity, inhibition of BEGF

stimulated HUVEC proliferation mediated by FLK-1/KDR activation, and inhibition of TNF- α production from murine bone marrow derived macrophages mediated by PYK2 activation. Finally, in a rodent collagen induced arthritis model, which mimics the histological and pathological changes associated with human RA, these compounds are efficacious in inhibiting joint swelling when dosed daily from the time of collagen immunization.

The KDR/FLK-1 Receptor and VEGF

Normal vasculogenesis and angiogenesis play important roles in a variety of physiological processes such as embryonic development, wound healing, organ regeneration and female reproductive processes such as follicle development in the corpus luteum during ovulation and placental growth after pregnancy. Folkman and Shing, 1992, *J. Biological Chem.* 267:10931-34. However, many diseases are driven by persistent unregulated or inappropriate angiogenesis. For example, in arthritis, new capillary blood vessels invade the joint and destroy the cartilage. In diabetes, new capillaries in the retina invade the vitreous, bleed and cause blindness. Folkman, 1987, in: *Congress of Thrombosis and Haemostasis* (Verstraete, et. al, eds.), Leuven University Press, Leuven, pp.583-596. Ocular neovascularization is the most common cause of blindness and dominates approximately twenty (20) eye diseases.

Moreover, vasculogenesis and/or angiogenesis have been associated with the growth of malignant solid tumors and metastasis. A tumor must continuously stimulate the growth of new capillary blood vessels for the tumor itself to grow. Furthermore, the new blood vessels embedded in a tumor provide a gateway for tumor cells to enter the circulation and to metastasize to distant sites in the body. Folkman, 1990, *J. Natl. Cancer Inst.* 82:4-6; Klagsbrunn and Soker, 1993, *Current Biology* 3:699-702; Folkman, 1991, *J. Natl., Cancer Inst.* 82:4-6; Weidner et al., 1991, *New Engl. J. Med.* 324:1-5.

Several polypeptides with *in vitro* endothelial cell growth promoting activity have been identified. Examples include acidic and basic fibroblastic growth factor (aFGF, bFGF), vascular endothelial growth factor (VEGF) and placental growth factor. Unlike aFGF and bFGF, VEGF has recently been reported to be an

endothelial cell specific mitogen. Ferrara and Henzel, 1989, *Biochem. Biophys. Res. Comm.* 161:851-858; Vaisman *et al.*, 1990, *J. Biol. Chem.* 265:19461-19566.

Thus, the identification of the specific receptors to which VEGF binds is an important advancement in the understanding of the regulation of endothelial cell proliferation. Two structurally closely related RTKs have been identified to bind VEGF with high affinity: the flt-1 receptor (Shibuya *et al.*, 1990, *Oncogene* 5:519-524; De Vries *et al.*, 1992, *Science* 255:989-991) and the KDR/FLK-1 receptor, discussed in the U.S. Patent Application No. 08/193,829. Consequently, it had been surmised that these RTKs may have a role in the modulation and regulation of endothelial cell proliferation.

Evidence, such as the disclosure set forth in copending U.S. Application Serial No. 08/193,829, strongly suggests that VEGF is not only responsible for endothelial cell proliferation, but also is a prime regulator of normal and pathological angiogenesis. *See generally*, Klagsburn and Soker, 1993, *Current Biology* 3:699-702; Houck *et al.*, 1992, *J. Biol. Chem.* 267:26031-26037. Moreover, it has been shown that KDR/FLK-1 and flt-1 are abundantly expressed in the proliferating endothelial cells of a growing tumor, but not in the surrounding quiescent endothelial cells. Plate *et al.*, 1992, *Nature* 359:845-848; Shweiki *et al.*, 1992, *Nature* 359:843-845.

Identification Of Agonists And Antagonists To The KDR/FLK-1 Receptor

In view of the deduced importance of RTKs in the control, regulation and modulation of endothelial cell proliferation and potentially vasculogenesis and/or angiogenesis, many attempts have been made to identify RTK "inhibitors" using a variety of approaches. These include the use of mutant ligands (U.S. Patent No. 4,966,849); soluble receptors and antibodies (Application No. WO 94/10202; Kendall and Thomas, 1994, *Proc. Natl. Acad. Sci. USA* 90:10705-10709; Kim *et al.*, 1993, *Nature* 362:841-844); and RNA ligands (Jellinek *et al.*, 1994, *Biochemistry* 33:10450-10456).

Furthermore, kinase inhibitors (WO 94/03427; WO 92/21660; WO 91/15495; WO 94/14808; U.S. Patent No. 5,330,992; Mariani *et al.*, 1994, *Proc. Am. Assoc. Cancer Res.* 35:2268), and inhibitors acting on receptor kinase signal

transduction pathways, such as protein kinase C inhibitors have been identified (Schuchter *et al.*, 1991, *Cancer Res.* 51:682-687); Takano *et al.*, 1993, *Mol. Bio. Cell* 4:358A; Kinsella *et al.*, 1992, *Exp. Cell Res.* 199:56-62; Wright *et al.*, 1992, *J. Cellular Phys.* 152:448-57).

5 More recently, attempts have been made to identify small molecules which act as kinase inhibitors for use in the treatment of cancer. Consequently, there is an unmet need for the identification and generation of effective small compounds which selectively inhibit the signal transduction of the KDR/FLK-1 receptor in order to effectively and specifically suppress vasculogenesis.

10 Some of the compounds of the present invention demonstrate excellent activity in biological assays and thus these compounds and related compounds are expected to be effective in treating Flk related disorders such as those driven by persistent unregulated or inappropriate angiogenesis.

15 Pharmaceutical Formulations And Administration

The compounds described herein can be administered to a human patient *per se*, or in pharmaceutical compositions where they are mixed with other active ingredients, as in combination therapy, or suitable carriers or excipient(s). Techniques for formulation and administration of the compounds of the instant application may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition.

a) Routes Of Administration

Suitable routes of administration may, for example, include oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including
25 intramuscular, subcutaneous, intravenous, intramedullary injections, as well as intrathecal, direct intraventricular, intraperitoneal, intranasal, or intraocular injections.

Alternately, one may administer the compound in a local rather than systemic manner, for example, via injection of the compound directly into a solid tumor, often
30 in a depot or sustained release formulation.

Furthermore, one may administer the drug in a targeted drug delivery system,

for example, in a liposome coated with tumor-specific antibody. The liposomes will be targeted to and taken up selectively by the tumor.

b) Composition/Formulation

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, *e.g.*, by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

Pharmaceutical preparations for oral use can be obtained by mixing one or more solid excipient with one or more compound of the invention, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or

polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection

may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

5 Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes.

10 Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

15 Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

20 In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for
25 example, as a sparingly soluble salt.

A pharmaceutical carrier for the hydrophobic compounds of the invention is a co-solvent system comprising benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. The co-solvent system may be the
30 VPD co-solvent system. VPD is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant Polysorbate 80, and 65% w/v polyethylene glycol 300, made up

to volume in absolute ethanol. The VPD co-solvent system (VPD:D5W) consists of VPD diluted 1:1 with a 5% dextrose in water solution. This co-solvent system dissolves hydrophobic compounds well, and itself produces low toxicity upon systemic administration. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied: for example, other low-toxicity nonpolar surfactants may be used instead of Polysorbate 80; the fraction size of polyethylene glycol may be varied; other biocompatible polymers may replace polyethylene glycol, *e.g.*, polyvinyl pyrrolidone; and other sugars or polysaccharides may substitute for dextrose.

Alternatively, other delivery systems for hydrophobic pharmaceutical compounds may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

Many of the PTK modulating compounds of the invention may be provided as salts with pharmaceutically compatible counterions. Pharmaceutically compatible salts may be formed with many acids, including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, maleic, succinic, etc. Salts tend to be more soluble in aqueous or other protic solvents than are the corresponding free base forms.

c) Effective Dosage.

Pharmaceutical compositions suitable for use in the present invention include compositions where the active ingredients are contained in an amount effective to achieve its intended purpose. More specifically, a therapeutically effective amount means an amount of compound effective to prevent, alleviate or ameliorate

symptoms of disease or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any compound used in the methods of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC_{50} as determined in cell culture (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of the PTK activity). Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the compounds described herein can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD_{50} and ED_{50} . Compounds which exhibit high therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See *e.g.*, Fingl *et al.*, 1975, in "The Pharmacological Basis of Therapeutics," Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the kinase modulating effects, or minimal effective concentration (MEC). The MEC will vary for each compound but can be estimated from *in vitro* data; *e.g.*, the concentration necessary to achieve 50-90% inhibition of the kinase using the assays described herein. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. However, HPLC assays or bioassays can be used to determine plasma concentrations.

Dosage intervals can also be determined using MEC value. Compounds should be administered using a regimen which maintains plasma levels above the MEC for 10-90% of the time, preferably between 30-90% and most preferably between 50-90%.

5 In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

10 Methods of preparing pharmaceutical formulations of the compounds, methods of determining the amounts of compounds to be administered to a patient, and modes of administering compounds to an organism are also disclosed in U.S. Patent Nos. 5,792,783, 5,880,141, 5,786,488, 5,834,504, 5,880,141, 5,883,113, 5,883,116, 5,886,020, and International patent publication numbers WO 96/22976
15 and WO 98/50356, all of which are incorporated herein by reference in their entirety, including any drawings. Those skilled in the art will appreciate that such descriptions are applicable to the present invention and can be easily adapted to it.

d) Packaging

The compositions may, if desired, be presented in a pack or dispenser device
20 which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accompanied with a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture,
25 use, or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the polynucleotide for human or veterinary administration. Such notice, for example, may be the labeling approved by the U.S. Food and Drug Administration for prescription drugs, or the approved product insert. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical
30 carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition. Suitable conditions indicated on the label may

include treatment of a tumor, inhibition of angiogenesis, treatment of fibrosis, diabetes, and the like.

Testing

5 The compounds of the present invention were tested for their ability to inhibit most of protein kinase activity. The biological assays and results of these inhibition studies are reported herein. Some of the methods used to measure modulation of protein kinase function are similar to those described in the International Publication No. WO 98/07695, published March 26, 1998, by Tang, et
10 al. and entitled "Indolinone Combinatorial Libraries and Related Products and Methods for the Treatment of Disease" (Lyon & Lyon Docket No. 221/187-PCT) and U.S. Application Serial No. 08/702,232, by Tang et al., and entitled "Indolinone Combinatorial Libraries and Related Products and Methods for the Treatment of Disease," filed August 23, 1996, with respect to the high throughput aspect of the
15 method. Both the 08/702,232 application and the International Publication No. WO 98/07695 are incorporated herein by reference in their entirety, including any drawings.

EXAMPLES

20 The examples below are non-limiting and are merely representative of various aspects and features of the present invention. The examples describe methods for synthesizing compounds of the invention and methods for measuring an effect of a compound on the function of protein kinases.

 The cells used in the methods are commercially available. The nucleic acid
25 vectors harbored by the cells are also commercially available and the sequences of genes for the various protein kinases are readily accessible in sequence data banks. Thus, a person of ordinary skill in the art can readily recreate the cell lines in a timely manner by combining the commercially available cells, the commercially available nucleic acid vectors, and the protein kinase genes using techniques readily
30 available to persons of ordinary skill in the art.

SYNTHETIC PROCEDURESEXAMPLE 1: PROCEDURE FOR SYNTHESIZING THE COMPOUNDS
OF THE INVENTION

5

The compounds of the present invention can be synthesized using the following general procedure.

General procedure for condensation of oxindole and aldehyde

- 10 To a mixture of oxindole (1 equiv.) and aldehyde (1-1.2 equiv.) in ethanol (0.1M) was added piperidine (1 eq.). The mixture was heated in an 90 °C oil bath for 1-3 hr. The precipitate was collected by vacuum filtration, washed with ethanol and dried to give (28-97% yield) of the desired product.

15 Preparation of 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde

- 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU, 8.39 mL, 56.1 mmol) was added to a stirred mixture of tosylmethyl isocyanide (10.95 g, 56.1 mmol) in tetrahydrofuran (THF, 56 mL) at 0 °C. After 15 minutes, to the mixture was added 5,6-dihydro-2*H*-pyran-2-one (5 g, 51 mmol). The mixture was then stirred at room temperature for 2 hours. The reaction was quenched with brine and extracted with THF several times. The combined extracts were dried (sodium sulfate), filter and concentrated to give 6,7-dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one.
- 20

- ¹H NMR (300 MHz, DMSO-*d*₆) δ 11.49 (br s, 1H, NH), 7.41 (m, 1H), 6.67 (m, 1H), 4.32 (t, *J* = 5.9 Hz, 2H, CH₂), 2.73 (t, *J* = 5.9 Hz, 2H, CH₂). MS 138 [M⁺+1].
- 25

- 6,7-Dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one (5 g, 36 mmol) was formulated under standard Vilsmeier reaction using *N,N*-dimethylformamide (DMF, 1.2 eq.) and phosphorus oxychloride (POCl₃, 1.1 eq.) in dichloromethane. The above mixture was stirred at room temperature for 1 hour. The precipitate salt was filtered, washed with dichloromethane, suspended in water and made basic with 6 N sodium hydroxide to pH = 12. The mixture was stirred for 5 minutes and filtered. The solid
- 30

was washed with ethanol/water mixture (1:1) to give 3.5 g (58%) of 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde.

¹H NMR (300 MHz, DMSO-*d*₆) δ 12.73 (br s, 1H, NH), 9.63 (s, 1H, CHO), 7.78 (s, 1H), 4.44 (t, *J* = 6.08 Hz, 2H, CH₂CH₂O), 3.10 (t, *J* = 6.08 Hz, 2H, CH₂CH₂O). MS 166 [M⁺+1].

Preparation of 4-oxo-4,5,6,7-tetrahydro-2H-pyrrolo[3,4-*c*]pyridine-1-carbaldehyde

2-Oxo-piperidine-1-carboxylic acid *tert*-butyl ester (3.8 g, 19 mmol) in THF (12 mL) was added dropwise to a cooled lithium diisopropylamide (LDA, 19 mL, 2.0 M solution in heptane/THF/ethylbenzene) at -78 °C. After stirring for 35 minutes at -78 °C, to the mixture was added dropwise a solution of phenyl disulfide (4.15 g, 19 mmol) and hexamethylphosphoramide (HMPA, 3.3 mL, 19 mmol) in THF (10 mL). The mixture was stirred at -78 °C and then gradually warmed up to room temperature. The reaction was poured into water (200 mL) and extracted with ether (3x). The combined ethereal extracts were washed consecutively with 10% NaOH and water, and were then dried and concentrated to give 2-oxo-3-phenylsulfanyl-piperidine-1-carboxylic acid *tert*-butyl ester as an oil. The oil was used in the next step without further purification.

3-Chloroperoxybenzoic acid (mCPBA, 4.7 g, 70%, 1 equivalent) was added portionwise to a cooled (0 °C) solution of the above 2-oxo-3-phenylsulfanyl-piperidine-1-carboxylic acid *tert*-butyl ester in dichloromethane (100 mL) and saturated sodium bicarbonate (NaHCO₃, 20 mL). The mixture was allowed to warm up slowly to room temperature and stirred until the reaction was completed by TLC. The reaction mixture was then extracted with dichloromethane, washed with NaHCO₃ (sat.) and dried to give 3-benzenesulfonyl-2-oxo-piperidine-1-carboxylic acid *tert*-butyl ester as an oil. It was use directly in the next step.

3-Benzenesulfonyl-2-oxo-piperidine-1-carboxylic acid *tert*-butyl ester from above in toluene (50 mL) was heated at 80 °C for one hour. The reaction mixture was concentrated and the resulting oil was purified by column chromatography (20-30% EtOAc in Hexane) to give 2g of 6-oxo-3,6-dihydro-2H-pyridine-1-carboxylic acid *tert*-butyl ester as an oil.

¹H NMR (360 MHz, DMSO-d₆) δ 6.91 (dt, 1H, COCH=CHCH₂), 5.81 (dt, *J* = 1.8 & 9.3 Hz, 1H, COCH=CHCH₂), 3.72 (t, *J* = 6.4 Hz, 2H, CONCH₂), 2.38 (m, 2H, CONCH₂CH₂), 1.44 (s, 9H, 3xCH₃).

DBU (1.65 mL, 11 mmol) was added to a stirred solution of tosylmethyl isocyanide (2.18 g, 11 mmol) in THF (11 mL) at 0 °C. After stirring for 15 minutes, 6-oxo-3,6-dihydro-2*H*-pyridine-1-carboxylic acid *tert*-butyl ester (2 g, 10 mmol) from above was added and the mixture was stirred at room temperature for 4 hours. The reaction mixture was quenched with saturated NaCl and extracted with THF. The combined extracts were dried and concentrated to give 2 g (85%) of 4-oxo-2,4,6,7-tetrahydro-pyrrolo[3,4-*c*]pyridine-5-carboxylic acid *tert*-butyl ester as a yellow solid.

¹H NMR (300 MHz, DMSO-d₆) δ 11.38 (br s, 1H, NH), 7.34 (m, 1H), 6.61 (s, 1H), 3.81 (t, *J* = 6.0 Hz, 2H, CH₂), 2.67 (t, *J* = 6.0 Hz, 2H CH₂), 1.44 (s, 9H, 3xCH₃).

MS APCI neg. 235 [M⁻-1].

POCl₃ (0.646 mL, 6.93 mmol) was added dropwise to the ice-cooled DMF (1.5 mL, 18.9 mmol). After stirring at room temperature for 30 minutes, the mixture was re-cooled to -5 °C and to it was added a solution of the above 4-oxo-2,4,6,7-tetrahydro-pyrrolo[3,4-*c*]pyridine-5-carboxylic acid *tert*-butyl ester (1.5 g, 6.3 mmol) in DMF (3 mL). The mixture was stirred at room temperature for 6 hours. The reaction mixture was quenched with ice cubes followed by the addition of 10 N potassium hydroxide, adjusting pH to 11-12. After stirring for 30 minutes, the mixture was extracted with ethyl acetate. The combined extracts were washed with brine, dried, and concentrated to give 1.2 g of 1-formyl-4-oxo-2,4,6,7-tetrahydro-pyrrolo[3,4-*c*]pyridine-5-carboxylic acid *tert*-butyl ester.

A mixture of 1-formyl-4-oxo-2,4,6,7-tetrahydro-pyrrolo[3,4-*c*]pyridine-5-carboxylic acid *tert*-butyl ester (1.2 g, 4.54 mmol) in 50% of trifluoroacetic acid in dichloromethane was stirred at room temperature for 30 minutes. The reaction was concentrated and the residue was recrystallized from dichloromethane to give 400 mg (75%) of 4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridine-1-carbaldehyde.

¹H NMR (360 MHz, DMSO-d₆) δ 12.30 (br s, 1H, NH), 9.60 (s, 1H, CHO),

7.46 (s, 1H), 7.34 (br s, 1H, NH), 3.36 (t, $J = 6.5$ Hz, 2H, CH_2), 2.95 (t, $J = 6.5$ Hz, 2H, CH_2).

MS 165 $[\text{M}^{++1}]$.

5 Preparation of 5-Methyl-4-oxo-4,5,6,7-tetrahydro-2H-pyrrolo[3,4-c]pyridine-1-carbaldehyde

1-Methyl-2-piperidone (4.54 mL, 40 mmol) in THF (25 mL) was added dropwise to the cooled lithium diisopropylamide (LDA, 40 mL, 2.0 M solution in heptane/THF/ethylbenzene) at -78°C . After stirring for 35 minutes at -78°C , to
10 the mixture was added dropwise a solution of phenyl disulfide (8.8 g, 40 mmol) and hexamethylphosphoramide (HMPA, 7 mL, 40 mmol) in THF (20 mL). The mixture was stirred at -78°C and then gradually warmed up to room temperature. The reaction was poured into water (400 mL) and extracted with ether (3x150 mL). The combined ethereal extracts were washed consecutively with 10% NaOH, water, 10%
15 HCl and then water, dried and concentrated to give 10 g ($>100\%$ crude yield) of 3-benzenesulfonyl-1-methyl-piperidin-2-one as an oil. The oil was used in the next step without further purification.

MS APCI +ve 222 $[\text{M}^++1]$.

3-Chloroperoxybenzoic acid (mCPBA, 4.9 g, 70%, 1 equivalent) was added
20 portionwise to a cooled (0°C) solution of 1-methyl-3-phenylsulfanyl-piperidin-2-one (5 g, 22.6 mmol) in dichloromethane (100 mL) and saturated NaHCO_3 (aq.) (20 mL). The mixture was allowed to warm up slowly to room temperature and stirred until the reaction was completed by TLC. The reaction mixture was then extracted with dichloromethane, washed with saturated sodium bicarbonate and dried to give
25 4.7 g (82% crude yield) of 3-benzenesulfonyl-1-methyl-piperidin-2-one as an oil.

A mixture of 3-benzenesulfonyl-1-methyl-piperidin-2-one from the previous step in toluene (50 mL) was heated to 80°C for one hour. The reaction mixture was concentrated and the residue was purified by column chromatography (80% EtOAc in Hex) to give 1.1 g (53% crude yield) of 1-methyl-5,6-dihydro-1H-pyridin-2-one
30 as a liquid.

^1H NMR (360 MHz, $\text{DMSO}-d_6$) δ 6.60 (dt, 1H, $\text{COCH}=\text{CHCH}_2$), 5.72 (dt, J

= 1.8 & 9.7 Hz, 1H, COCH=CHCH₂), 3.35 (t, *J* = 7.2 Hz, 2H, CONCH₂), 2.82 (s, 3H, CH₃), 2.33 (m, 2H, CONCH₂CH₂), 1.44 (s, 9H, 3xCH₃).

MS APCI +ve 112 [M⁺+1].

To a stirred solution of lithium bis(trimethylsilyl)amide (11 mL of 1 M solution in THF) cooled to -78 °C under nitrogen was added dropwise a solution of tosylmethyl isocyanide (1.9 g, 10 mmol) in THF (45 mL). After stirring for 40 minutes at -78 °C, a solution of 1-methyl-5,6-dihydro-1*H*-pyridin-2-one (1.1 g, 10 mmol) in THF (10 mL) was added and the mixture was stirred at room temperature for overnight. The reaction was concentrated and the residue was partitioned between water (150 mL) and dichloromethane (150 mL). The aqueous was extracted with dichloromethane (3x). The combined organic layers were concentrated and dried under high vacuum. The resulting foam was recrystallized from dichloromethane to give 633 mg (42%) of 5-methyl-2,5,6,7-tetrahydro-pyrrolo[3,4-*c*]pyridin-4-one as a light yellow solid.

¹HNMR (360 MHz, DMSO-*d*₆) δ 11.0 (br s, 1H, NH), 7.08 (s, 1H), 6.53 (s, 1H), 3.42 (t, *J* = 6.6 Hz, 2H, CH₂), 2.89 (s, 3H, CH₃), 2.70 (t, *J* = 6.6 Hz, 2H CH₂).

5-Methyl-2,5,6,7-tetrahydro-pyrrolo[3,4-*c*]pyridin-4-one was formylated under standard Vilsmeier conditions using DMF (3 equiv.) and POCl₃ (1.1 equiv.) to give 250 mg (33%) of 5-methyl-4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridine-1-carbaldehyde.

¹HNMR (360 MHz, DMSO-*d*₆) δ 12.37 (br s, 1H, NH), 9.60 (s, 1H, CHO), 7.45 (s, 1H), 3.52 (t, *J* = 6.6 Hz, 2H, CH₂), 3.04 (t, *J* = 6.6 Hz, 2H, CH₂), 2.92 (s, 3H, CH₃).

MS *m/z* 179 [M⁺+1].

25

Compound IN-001:

4-Methyl-2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrol-1-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid methylamide

30

4-Methyl-2-oxo-2,3-dihydro-1*H*-indole-5-sulfonic acid methylamide (0.2 g, 0.83 mmol) was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-

carbaldehyde (1.2 eq, 0.17g) to give 0.31 g (97%) of the title compound as a yellow solid.

¹H NMR (300 MHz, DMSO-d₆) δ 13.77 (s, 1H, NH), 11.42 (s, 1H, NH-CO), 7.92 (d, *J* = 3.3 Hz, 1H), 7.72 (d, *J* = 8.1 Hz, ArH), 7.69 (s, 1H), 7.42 (q, *J* = 4.5 Hz, 1H, SO₂NH), 6.89 (d, *J* = 8.5 Hz, 1H), 4.49 (t, *J* = 5.8 Hz, 2H, CO₂CH₂CH₂), 3.08 (t, *J* = 5.8 Hz, 2H, CO₂CH₂CH₂), 2.84 (s, 3H, CH₃), 2.42 (d, *J* = 4.7 Hz, 3H, SO₂NHCH₃). MS 388 [M⁺+1].

Compound IN-002:

10 2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrol-1-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid methylamide

2-Oxo-2,3-dihydro-1*H*-indole-5-sulfonic acid methylamide (0.2 g, 0.88 mmol) was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde (1.2 eq, 0.18 g) to give 0.26 g (79%) of the title compound as a yellow solid.

¹H NMR (300 MHz, DMSO-d₆) δ 13.74 (s, 1H, NH), 11.45 (s, 1H, NH-CO), 8.28 (d, *J* = 1.9 Hz, 1H), 8.0 (s, 1H, C=CH), 7.95 (d, *J* = 3.2 Hz, 1H), 7.60 (dd, *J* = 1.9 and 8.3 Hz, 1H), 7.21 (m, 1H, SO₂NH), 7.05 (d, *J* = 8.2 Hz, 1H), 4.50 (t, *J* = 5.8 Hz, 2H, CO₂CH₂CH₂), 3.18 (t, *J* = 5.8 Hz, 2H, CO₂CH₂CH₂), 2.41 (d, *J* = 5.2 Hz, 3H, SO₂NHCH₃). MS 374 [M⁺+1].

Compound IN-003:

25 1-(4-Methyl-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one

4-Methyl-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 295.2 [M⁺+1].

Compound IN-004:

1-(5-Methoxy-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one

5 5-Methoxy-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 311.2 [$M^+ + 1$].

Compound IN-005:

10 1-(6-Methoxy-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one

6-Methoxy-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrole-1-carbaldehyde to give the title compound.

15 MS *m/z* 311.2 [$M^+ + 1$].

Compound IN-006:

1-(5-Bromo-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one

20

5-Bromo-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 359.2 & 361.1 [$M^+ + 1$].

25 Compound IN-007:

1-(5-Chloro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one

5-Chloro-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrole-1-carbaldehyde to give the title compound.

30

MS *m/z* 315.2 [$M^+ + 1$].

Compound IN-008:

1-[4-(2-Hydroxy-ethyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one

5

4-(2-Hydroxy-ethyl)-1,3-dihydro-indol-2-one (0.1 g, 0.6 mmol) was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde (1.2 eq, 0.06 g) to give 0.5 g (28%) of the title compound as a yellow solid.

¹H NMR (300 MHz, DMSO-*d*₆) δ 13.87 (s, 1H, NH), 11.07 (s, 1H, NH-CO),
10 7.90 (d, *J* = 3.0Hz, 1H), 7.55 (s, 1H, C=CH), 7.09 (m, 1H), 6.84 (d, *J* = 7.6Hz, 1H),
6.76 (d, *J* = 7.7Hz, 1H), 4.89 (t, *J* = 4.8Hz, 1H, CH₂CH₂OH), 4.49 (t, *J* = 6.0Hz, 2H,
CO₂CH₂CH₂pyrrole), 3.70 (m, 2H, HOCH₂CH₂Ar and CO₂CH₂CH₂pyrrole). MS *m/z*
325.2 [*M*⁺+1].

15 Compound IN-009:

1-(2-Oxo-5-phenyl-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one

5-Phenyl-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.
20

MS *m/z* 357.2 [*M*⁺+1].

Compound IN-010:

25 2-Oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrol-1-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid amide

2-Oxo-2,3-dihydro-1*H*-indole-5-sulfonic acid amide was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title
30 compound.

MS *m/z* 360.2 [*M*⁺+1].

Compound IN-011:

2-Oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrol-1-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid dimethylamide

- 5 2-Oxo-2,3-dihydro-1*H*-indole-5-sulfonic acid dimethylamide was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 388.2 [*M*⁺+1].

10 Compound IN-012:

2-Oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrol-1-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid isopropylamide

- 2-Oxo-2,3-dihydro-1*H*-indole-5-sulfonic acid isopropylamide was condensed
15 with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 402.2 [*M*⁺+1].

Compound IN-013:

- 20 2-Oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrol-1-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid phenylamide

- 2-Oxo-2,3-dihydro-1*H*-indole-5-sulfonic acid phenylamide was condensed
with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title
25 compound.

MS *m/z* 436.2 [*M*⁺+1].

Compound IN-014:

- N*-[2-Oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrol-1-ylmethylene)-
30 2,3-dihydro-1*H*-indol-6-yl]-acetamide

N-(2-Oxo-2,3-dihydro-1*H*-indol-6-yl)-acetamide was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 338.2 [*M*⁺+1].

5 Compound IN-015:

1-(2-Oxo-6-phenyl-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one

6-Phenyl-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 357.2 [*M*⁺+1].

Compound IN-016:

15 1-(5-Fluoro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one

5-Fluoro-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 299.2 [*M*⁺+1].

20

Compound IN-017:

2-Oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrol-1-ylmethylene)-2,3-dihydro-1*H*-indole-5-carboxylic acid

25 2-Oxo-2,3-dihydro-1*H*-indole-5-carboxylic acid was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 325.2 [*M*⁺+1].

Compound IN-018:

30 2-Oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrol-1-ylmethylene)-2,3-dihydro-1*H*-indole-6-carboxylic acid

2-Oxo-2,3-dihydro-1*H*-indole-6-carboxylic acid was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 325.2 [*M*⁺+1].

5

Compound IN-019:

1-(6-Chloro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one

10 6-Chloro-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 315.2 [*M*⁺+1].

Compound IN-020:

15 1-(6-Bromo-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one
one

6-Bromo-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

20

MS *m/z* 359.2 & 361 [*M*⁺+1].

Compound IN-021:

1-[6-(2-Methoxy-phenyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one

25

6-(2-Methoxy-phenyl)-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 387.2 [*M*⁺+1].

30

Compound IN-022:

1-[6-(3-Methoxy-phenyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one

5 6-(3-Methoxy-phenyl)-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 387.2 [$M^+ + 1$].

Compound IN-023:

10 1-[6-(4-Methoxy-phenyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one

6-(4-Methoxy-phenyl)-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

15 MS *m/z* 387.2 [$M^+ + 1$].

Compound IN-024:

1-[6-(4-Fluoro-phenyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one

20

6-(4-Fluoro-phenyl)-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 375.2 [$M^+ + 1$].

25 Compound IN-025:

1-(2-Oxo-6-pyridin-3-yl-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one

30 6-Pyridin-3-yl-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 358.4 [$M^+ + 1$].

Compound IN-026:

1-[5-(2,3-Dihydro-indole-1-sulfonyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one

- 5 To a mixture of 5-chlorosulfonyl-2-oxindole (5 g, 21.6 mmol) and indoline (2.9 mL, 26 mmol) in THF (20 mL) was added pyridine (3.4 g, 43 mmol). After stirring at room temperature for one day, the precipitate was collected by filtration, washed with water in ethanol (20%), dried, washed with 60 mL of hot ethanol and dried under vacuum to give 7.5 g (over 100%) of 5-(2,3-dihydro-indole-1-sulfonyl)-
10 1,3-dihydro-indol-2-one as a pink solid.

¹H NMR (360 MHz, DMSO-*d*₆) δ 10.76 (br s, 1H, NH), 7.62 (m, 2H), 7.43 (d, *J* = 8.0 Hz, 1H), 7.13-7.18 (m, 2H), 6.95 (dt, *J* = 0.9 & 7.5 Hz, 1H), 6.90 (d, *J* = 8.0 Hz, 1H), 3.87 (t, *J* = 8.5 Hz, 2H, CH₂CH₂), 3.51 (s, 2H, CH₂), 2.91 (t, *J* = 8.5 Hz, 2H, CH₂CH₂). MS-EI 314 [M⁺].

- 15 5-(2,3-Dihydro-indole-1-sulfonyl)-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 462.3 [M⁺+1].

20 Compound IN-027:

1-[5-(3,4-Dihydro-2*H*-quinoline-1-sulfonyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one

- 5-(3,4-Dihydro-2*H*-quinoline-1-sulfonyl)-1,3-dihydro-indol-2-one was
25 condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 476.2 [M⁺+1].

Compound IN-028:

- 30 1-[5-(3,4-Dihydro-1*H*-isoquinoline-2-sulfonyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one

5-(3,4-Dihydro-1*H*-isoquinoline-2-sulfonyl)-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 476.3 [M^+ +1].

5

Compound IN-029:

1-[5-(5-Bromo-2,3-dihydro-indole-1-sulfonyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one

10 5-(5-Bromo-2,3-dihydro-indole-1-sulfonyl)-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 540.3 & 542 [M^+ +1].

15 Compound IN-030:

2-Oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrol-1-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid (3-chloro-phenyl)-methyl-amide

20 2-Oxo-2,3-dihydro-1*H*-indole-5-sulfonic acid (3-chloro-phenyl)-methyl-amide was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 484.2 [M^+ +1].

Compound IN-031:

25 1-(6-Fluoro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one

6-Fluoro-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

30 MS *m/z* 299.2 [M^+ +1].

Compound IN-032:

1-(5-Chloro-4-methyl-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one

5 5-Chloro-4-methyl-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 329.2 [*M*⁺+1].

Compound IN-033:

10 1-(7-Chloro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one

7-Chloro-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

15 MS *m/z* 315.2 [*M*⁺+1].

Compound IN-034:

3-[2-Oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrol-1-ylmethylene)-2,3-dihydro-1*H*-indol-5-yl]-benzoic acid

20

3-(2-Oxo-2,3-dihydro-1*H*-indol-5-yl)-benzoic acid was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS *m/z* 401.2 [*M*⁺+1].

25

Compound IN-035:

3-[2-Oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrol-1-ylmethylene)-2,3-dihydro-1*H*-indol-6-yl]-benzoic acid

30

3-(2-Oxo-2,3-dihydro-1*H*-indol-6-yl)-benzoic acid was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title

compound.

MS m/z 401.2 [$M^+ + 1$].

Compound IN-036:

5 2-Oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrol-1-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid (3-chloro-phenyl)-amide

2-Oxo-2,3-dihydro-1*H*-indole-5-sulfonic acid (3-chloro-phenyl)-amide was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to
10 give the title compound.

MS m/z 470.2 [$M^+ + 1$].

Compound IN-037:

1-(5-Bromo-4-methyl-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-
15 dihydro-2*H*-pyrano[3,4-*c*]pyrrol-4-one

5-Bromo-4-methyl-1,3-dihydro-indol-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS m/z 373.2 & 375 [$M^+ + 1$].
20

Compound IN-038:

1-(2-Oxo-5-phenyl-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-*c*]pyridin-4-one

25 A mixture of 5-phenyl-1,3-dihydro-indol-2-one (41.8 mg, 0.2 mmol), 4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridine-1-carbaldehyde (1 equivalent) and 0.1 mL of piperidine in ethanol (1 mL) was heated in a sealed tube at 80 °C for 3 hours. The precipitate was collected by vacuum filtration, washed with ethanol and dried to give the title compound as a yellow solid.

30 ¹H NMR (300 MHz, DMSO-*d*₆) δ 13.58 (s, 1H, NH), 11.06 (br s, 1H, NH), 8.16 (d, J = 1.3 Hz, 1H), 7.86 (s, 1H, H-vinyl), 7.68-7.71 (m, 3H), 7.40-7.48 (m,

4H), 7.31 (m, 1H), 6.95 (d, $J = 8.1$ Hz, 1H), 3.41 (m, 2H, CH₂), 3.02 (t, $J = 6.4$ Hz, 2H, CH₂). MS m/z 356 [$M^+ + 1$].

Compound IN-039:

- 5 1-(2-Oxo-6-phenyl-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-*c*]pyridin-4-one

A mixture of 6-phenyl-1,3-dihydro-indol-2-one (41.8 mg, 0.2 mmol), 4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridine-1-carbaldehyde (1 equivalent) and 0.1
10 mL of piperidine in ethanol (1 mL) was heated in a sealed tube at 80 °C for 3 hours. The precipitate was collected by vacuum filtration, washed with ethanol and dried to give the title compound as a yellow solid.

¹H NMR (300 MHz, DMSO-*d*₆) δ 13.55 (s, 1H, NH), 11.09 (br s, 1H, NH), 7.86 (d, $J = 7.7$ Hz, 1H), 7.72 (s, 1H, H-vinyl), 7.68 (d, $J = 3.4$ Hz, 1H), 7.64 (m,
15 2H), 7.45 (t, $J = 7.5$ Hz, 2H), 7.30-7.40 (m, 3H), 7.10 (d, $J = 1.2$ Hz, 1H), 3.41 (m, 2H, CH₂), 2.99 (t, $J = 6.7$ Hz, 2H, CH₂). MS m/z 356 [$M^+ + 1$].

Compound IN-040:

- 20 1-(6-Bromo-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-*c*]pyridin-4-one

A mixture of 6-bromo-1,3-dihydro-indol-2-one (42.4 mg, 0.2 mmol), 4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridine-1-carbaldehyde (1 equivalent) and 0.1 mL of piperidine in ethanol (1 mL) was heated in a sealed tube at 80 °C for 3 hours.
25 The precipitate was collected by vacuum filtration, washed with ethanol and dried to give the title compound as a yellow solid.

¹H NMR (300 MHz, DMSO-*d*₆) δ 13.45 (s, 1H, NH), 11.09 (br s, 1H, NH), 7.74 (d, $J = 8.4$ Hz, 1H), 7.74 (s, 1H, H-vinyl), 7.70 (d, $J = 3.3$ Hz, 1H), 7.41 (br s, 1H, NH), 7.19 (dd, $J = 1.4$ & 8.4 Hz, 1H), 7.01 (d, $J = 1.4$ Hz, 1H), 3.39 (m, 2H,
30 CH₂), 2.96 (t, $J = 6.4$ Hz, 2H, CH₂). MS m/z 358 & 360 [$M^+ + 1$].

Compound IN-041:

1-(6-Chloro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one

5 A mixture of 6-chloro-1,3-dihydro-indol-2-one (33.5 mg, 0.2 mmol), 4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridine-1-carbaldehyde (1 equivalent) and 0.1 mL of piperidine in ethanol (1 mL) was heated in a sealed tube at 80 °C for 3 hours. The precipitate was collected by vacuum filtration, washed with ethanol and dried to give the title compound as a yellow solid.

10 ¹H NMR (300 MHz, DMSO-*d*₆) δ 13.45 (s, 1H, NH), 11.10 (br s, 1H, NH), 7.80 (d, *J* = 8.1 Hz, 1H), 7.73 (s, 1H, H-vinyl), 7.68 (d, *J* = 3.2 Hz, 1H), 7.40 (br s, 1H, NH), 7.05 (dd, *J* = 1.8 & 8.1 Hz, 1H), 6.88 (d, *J* = 1.8 Hz, 1H), 3.40 (m, 2H, CH₂), 2.97 (t, *J* = 6.5 Hz, 2H, CH₂). MS *m/z* 314 [M⁺+1].

15 Compound IN-042:

2-Oxo-3-(4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridin-1-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid dimethylamide

20 A mixture of 2-oxo-2,3-dihydro-1*H*-indole-5-sulfonic acid dimethylamide (48 mg, 0.2 mmol), 4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridine-1-carbaldehyde (1 equivalent) and 0.1 mL of piperidine in ethanol (1 mL) was heated in a sealed tube at 80 °C for 3 hours. The precipitate was collected by vacuum filtration, washed with ethanol and dried to give the title compound as a yellow solid.

25 ¹H NMR (300 MHz, DMSO-*d*₆) δ 13.51 (s, 1H, NH), 11.25 (br s, 1H, NH), 8.29 (d, *J* = 1.6 Hz, 1H), 8.03 (s, 1H, H-vinyl), 7.73 (d, *J* = 3.2 Hz, 1H), 7.53 (dd, *J* = 1.6 & 8.2 Hz, 1H), 7.43 (br s, 1H, NH), 7.07 (d, *J* = 8.2 Hz, 1H), 3.43 (m, 2H, CH₂), 3.06 (t, *J* = 6.6 Hz, 2H, CH₂), 2.60 (s, 6H, 2xCH₃). MS *m/z* 387 [M⁺+1].

30 Compound IN-043:

2-Oxo-3-(4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridin-1-

ylmethylene)-2,3-dihydro-1*H*-indole-5-carboxylic acid dimethylamide

Benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP, 3.5 g, 6.72 mmol) was added to a mixture of 5-carboxy-2-oxindole (1 g, 5.6 mmol), dimethylamine (5.6 mL of 2.0 M in THF, 11.2 mmol) and triethylamine (2.0 mL, 14 mmol) in dichloromethane. After stirring at room temperature for 3 hours, the reaction was diluted with more dichloromethane, washed with water, saturated sodium bicarbonate and brine, dried and concentrated. The residue was purified by column chromatography to give 480 mg (42%) of 2-oxo-2,3-dihydro-1*H*-indole-5-carboxylic acid dimethylamide.

¹H NMR (300 MHz, DMSO-*d*₆) δ 10.50 (br s, 1H, NH), 7.23 (m, 2H), 6.81 (d, *J* = 6.9 Hz, 1H), 3.49 (s, 2H, CH₂), 2.93 (s, 6H, 2xCH₃). MS-EI *m/z* 204 [M⁺].

A mixture of 2-oxo-2,3-dihydro-1*H*-indole-5-carboxylic acid dimethylamide (40.8 mg, 0.2 mmol), 4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridine-1-carbaldehyde (1 eq.) and 0.1 mL of piperidine in ethanol (1 mL) was heated in a sealed tube at 80 °C for 3 hours. The precipitate was collected by vacuum filtration, washed with ethanol and dried to give the title compound as a yellow solid.

¹H NMR (300 MHz, DMSO-*d*₆) δ 13.53 (s, 1H, NH), 11.14 (br s, 1H, NH), 7.92 (d, *J* = 1.3 Hz, 1H), 7.82 (s, 1H, H-vinyl), 7.68 (d, *J* = 2.7 Hz, 1H), 7.40 (br s, 1H, NH), 7.20 (dd, *J* = 1.3 & 8.0 Hz, 1H), 6.89 (d, *J* = 8.0 Hz, 1H), 3.40 (m, 2H, CH₂), 2.99 (m, 2H, CH₂), 2.97 (s, 6H, 2xCH₃). MS 351 *m/z* [M⁺+1].

Compound IN-044:

1-[2-Oxo-5-(pyrrolidine-1-carbonyl)-1,2-dihydro-indol-3-ylidenemethyl]-2,5,6,7-tetrahydro-pyrrolo[3,4-*c*]pyridin-4-one

PyBOP (3.5 g, 6.72 mmol) was added to a mixture of 5-carboxy-2-oxindole (1 g, 5.6 mmol), pyrrolidine (0.5 mL, 6.2 mmol) and triethylamine (2.0 mL, 14 mmol) in dichloromethane. After stirring at room temperature for 4 hours, the reaction was diluted with more dichloromethane, washed with saturated sodium bicarbonate and brine, dried and concentrated. The residue was purified by column

chromatography to give 5-(pyrrolidine-1-carbonyl)-1,3-dihydro-indol-2-one.

¹H NMR (300 MHz, DMSO-d₆) δ 10.52 (br s, 1H, NH), 7.37 (m, 2H), 6.80 (d, *J* = 8.5 Hz, 1H), 3.49 (s, 2H, CH₂), 3.42 (m, 4H, 2xCH₂), 1.81 (m, 4H, 2xCH₂). MS-EI *m/z* 230 [M⁺].

- 5 A mixture of 5-(pyrrolidine-1-carbonyl)-1,3-dihydro-indol-2-one (46 mg, 0.2 mmol), 4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridine-1-carbaldehyde (1 equivalent) and 0.1 mL of piperidine in ethanol (1 mL) was heated in a sealed tube at 80 °C for 3 hours. The precipitate was collected by vacuum filtration, washed with ethanol and dried to give the title compound as a yellow solid.

- 10 ¹H NMR (300 MHz, DMSO-d₆) δ 13.52 (s, 1H, NH), 11.14 (br s, 1H, NH), 8.02 (d, *J* = 1.3 Hz, 1H), 7.83 (s, 1H, H-vinyl), 7.69 (d, *J* = 2.9 Hz, 1H), 7.40 (br s, 1H, NH), 7.32 (dd, *J* = 1.3 & 7.7 Hz, 1H), 6.89 (d, *J* = 7.7 Hz, 1H), 3.39-3.48 (m, 6H), 3.0 (t, *J* = 6.4 Hz, 2H, CH₂), 1.84 (m, 4H, 2xCH₂). MS *m/z* 377 [M⁺+1].

15 Compound IN-045:

1-[5-(Morpholine-4-carbonyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-
2,5,6,7-tetrahydro-pyrrolo[3,4-*c*]pyridin-4-one

- 20 PyBOP (3.5 g, 6.72 mmol) was added to a mixture of 5-carboxy-2-oxindole (1 g, 5.6 mmol), morpholine (0.5 mL, 6.2 mmol) and triethylamine (2.0 mL, 14 mmol) in dichloromethane. After stirring at room temperature for 4 hours, the reaction was diluted with more dichloromethane, washed with saturated sodium bicarbonate and brine, dried and concentrated. The residue was purified by column chromatography to give 5-(morpholine-4-carbonyl)-1,3-dihydro-indol-2-one.

- 25 ¹H NMR (300 MHz, DMSO-d₆) δ 10.54 (br s, 1H, NH), 7.24 (m, 2H), 6.83 (d, *J* = 7.6 Hz, 1H), 3.56 (m, 4H, 2xCH₂), 3.49 (s, 2H, CH₂), 3.47 (m, 4H, 2xCH₂). MS APCI neg. *m/z* 245 [M⁺-1].

- 30 A mixture of 5-(morpholine-4-carbonyl)-1,3-dihydro-indol-2-one (49.2 mg, 0.2 mmol), 4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridine-1-carbaldehyde (1 eq.) and 0.1 mL of piperidine in ethanol (1 mL) was heated in a sealed tube at 80 °C for 3 hours. The precipitate was collected by vacuum filtration, washed with ethanol

and dried to give the title compound as a yellow solid.

¹H NMR (300 MHz, DMSO-d₆) δ 13.52 (s, 1H, NH), 11.16 (br s, 1H, NH), 7.92 (d, *J* = 1.4 Hz, 1H), 7.83 (s, 1H, H-vinyl), 7.69 (d, *J* = 3.0 Hz, 1H), 7.41 (br s, 1H, NH), 7.21 (dd, *J* = 1.4 & 7.9 Hz, 1H), 6.91 (d, *J* = 7.9 Hz, 1H), 3.59 (m, 4H, 2xCH₂), 3.52 (m, 4H, 2xCH₂), 3.41 (m, 2H, CH₂), 3.0 (t, *J* = 6.6 Hz, 2H, CH₂). MS *m/z* 393 [M⁺+1].

Compound IN-046:

1-[4-(2-Hydroxy-ethyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-2,5,6,7-tetrahydro-pyrrolo[3,4-*c*]pyridin-4-one

A mixture of 4-(2-hydroxy-ethyl)-1,3-dihydro-indol-2-one (35.4 mg, 0.2 mmol), 4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridine-1-carbaldehyde (32.8 mg, 0.2 mmol) and 0.1 mL of piperidine in ethanol (1 mL) was heated in a sealed tube at 80 °C for 4 hours. The precipitate was collected by vacuum filtration, washed with cold ethanol and dried to give the title compound.

¹H NMR (300 MHz, DMSO-d₆) δ 13.62 (br s, 1H, NH), 11.0 (s, 1H, NH), 7.66 (d, *J* = 3.0 Hz, 1H), 7.55 (s, 1H, H-vinyl), 7.38 (br s, 1H, NH), 7.07 (t, *J* = 7.6 Hz, 1H), 6.83 (d, *J* = 7.6 Hz, 1H), 6.76 (d, *J* = 7.6 Hz, 1H), 4.89 (t, *J* = 4.7 Hz, 1H, OH), 3.70 (m, 2H, CH₂), 3.41 (m, 2H, CH₂), 3.06 (t, *J* = 7.2 Hz, 2H, CH₂), 2.89 (t, *J* = 6.4 Hz, 2H, CH₂). MS *m/z* 324 [M⁺+1].

Compound IN-047:

1-(5-Methoxy-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-*c*]pyridin-4-one

A mixture of 5-methoxy-1,3-dihydro-indol-2-one (32.6 mg, 0.2 mmol), 4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridine-1-carbaldehyde (32.8 mg, 0.2 mmol) and 0.1 mL of piperidine in ethanol (1 mL) was heated in a sealed tube at 80 °C for 4 hours. The precipitate was collected by vacuum filtration, washed with cold ethanol and dried to give the title compound.

¹H NMR (300 MHz, DMSO-d₆) δ 13.65 (br s, 1H, NH), 10.79 (s, 1H, NH), 7.70 (s, 1H, H-vinyl), 7.66 (d, *J* = 2.8 Hz, 1H), 7.47 (d, *J* = 2.4 Hz, 1H), 7.38 (br s, 1H, NH), 6.75 (m, 2H), 3.41 (m, 2H, CH₂), 3.32 (s, 3H, OCH₃), 2.99 (t, *J* = 6.5 Hz, 2H, CH₂). MS *m/z* 310 [M⁺+1].

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Compound IN-048:

1-(5-Fluoro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-*c*]pyridin-4-one

10

A mixture of 5-fluoro-1,3-dihydro-indol-2-one (30.2 mg, 0.2 mmol), 4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridine-1-carbaldehyde (32.8 mg, 0.2 mmol) and 0.1 mL of piperidine in ethanol (1 mL) was heated in a sealed tube at 80 °C for 4 hours. The precipitate was collected by vacuum filtration, washed with cold ethanol and dried to give the title compound.

15

¹H NMR (300 MHz, DMSO-d₆) δ 13.57 (br s, 1H, NH), 10.98 (s, 1H, NH), 7.77 (s, 1H, H-vinyl), 7.73 (dd, *J* = 2.6 & 9.3 Hz, 1H), 7.70 (d, *J* = 2.7 Hz, 1H), 7.40 (br s, 1H, NH), 6.96 (m, 1H), 6.84 (dd, 1H), 3.41 (m, 2H, CH₂), 2.98 (t, *J* = 6.5 Hz, 2H, CH₂). MS *m/z* 298 [M⁺+1].

20

Compound IN-049:

1-(5-Bromo-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-*c*]pyridin-4-one

25

A mixture of 5-bromo-1,3-dihydro-indol-2-one (23 mg, 0.11 mmol), 4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridine-1-carbaldehyde (29.7 mg, 0.18 mmol) and 0.1 mL of piperidine in ethanol (1 mL) was heated in a sealed tube at 80 °C for 4 hours. The precipitate was collected by vacuum filtration, washed with cold ethanol and dried to give 26 mg (67%) of the title compound.

30

¹H NMR (300 MHz, DMSO-d₆) δ 13.52 (br s, 1H, NH), 11.09 (br s, 1H, NH), 8.08 (d, *J* = 2.3 Hz, 1H), 7.83 (s, 1H, H-vinyl), 7.70 (d, *J* = 3.0 Hz, 1H), 7.41

(br s, 1H, NH), 7.29 (dd, $J = 2.3$ & 8.3 Hz, 1H), 6.82 (d, $J = 8.3$ Hz, 1H), 3.40 (m, 2H, CH₂), 2.99 (t, $J = 6.5$ Hz, 2H, CH₂). MS m/z 358/ 360

Compound IN-050:

5 2-Oxo-3-(4-oxo-4,5,6,7-tetrahydro-2H-pyrrolo[3,4-c]pyridin-1-ylmethylene)-2,3-dihydro-1H-indole-5-carboxylic acid

A mixture of 2-oxo-2,3-dihydro-1H-indole-5-carboxylic acid (44.5 mg, 0.25 mmol), 4-oxo-4,5,6,7-tetrahydro-2H-pyrrolo[3,4-c]pyridine-1-carbaldehyde (41 mg, 10 0.25 mmol) and 0.1 mL of piperidine in ethanol (1 mL) was heated in a sealed tube at 80 °C for 6 hours. The precipitate was collected by vacuum filtration, washed with cold ethanol. The solid was then dissolved in methanol, the insoluble materials were removed and the filtrate was concentrated to give 20 mg (25%) of the title compound as a yellow solid.

15 ¹H NMR (300 MHz, DMSO-d₆) δ 13.59 (s, 1H, NH), 11.10 (br s, 1H, NH), 8.24 (s, 1H), 7.76 (d, $J = 7.8$ Hz, 1H), 7.65 (m, 2H), 7.37 (br s, 1H, NH), 6.78 (d, $J = 8.1$ Hz, 1H), 3.38 (m, CH₂), 3.0 (t, 2H, CH₂). MS m/z 324 [$M^+ + 1$].

Compound IN-051:

20 2-Oxo-3-(4-oxo-4,5,6,7-tetrahydro-2H-pyrrolo[3,4-c]pyridin-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid amide

A mixture of 2-oxo-2,3-dihydro-1H-indole-5-sulfonic acid amide (63 mg, 0.3 mmol), 4-oxo-4,5,6,7-tetrahydro-2H-pyrrolo[3,4-c]pyridine-1-carbaldehyde (50 mg, 25 0.3 mmol) and 0.1 mL of piperidine in ethanol (1 mL) was heated in a sealed tube at 80 °C for 4 hours. The precipitate was collected by vacuum filtration, washed with cold ethanol and dried to give 89 mg (82%) of the title compound.

30 ¹H NMR (300 MHz, DMSO-d₆) δ 13.50 (s, 1H, NH), 11.22 (br s, 1H, NH), 8.27 (d, $J = 1.5$ Hz, 1H), 7.88 (s, 1H, H-vinyl), 7.72 (d, $J = 3.0$ Hz, 1H), 7.64 (dd, $J = 1.5$ & 8.0 Hz, 1H), 7.42 (br s, 1H, NH), 7.17 (br s, 2H, NH₂), 7.01 (d, $J = 8.0$ Hz, 1H), 3.40 (m, 2H, CH₂), 3.02 (t, $J = 6.6$ Hz, 2H, CH₂). MS m/z 359 [$M^+ + 1$].

Compound IN-052:

2-Oxo-3-(4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridin-1-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid methylamide

5 A mixture of 2-oxo-2,3-dihydro-1*H*-indole-5-sulfonic acid methylamide (66 mg, 0.3 mmol), 4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridine-1-carbaldehyde (50 mg, 0.3 mmol) and 0.1 mL of piperidine in ethanol (1 mL) was heated in a sealed tube at 80 °C for 4 hours. The precipitate was collected by vacuum filtration, washed with cold ethanol and dried to give 50 mg (45%) of the title compound.

10 ¹H NMR (300 MHz, DMSO-*d*₆) δ 13.51 (br s, 1H, NH), 11.2 (br s, 1H, NH), 8.26 (d, *J* = 1.3 Hz, 1H), 7.94 (s, 1H, H-vinyl), 7.73 (d, *J* = 3.1 Hz, 1H), 7.58 (dd, *J* = 1.3 & 8.0 Hz, 1H), 7.42 (br s, 1H, NH), 7.20 (m, 1H, CH₃NH), 7.05 (d, *J* = 8.0 Hz, 1H), 3.40 (m, 2H, CH₂), 3.04 (t, *J* = 6.5 Hz, 2H, CH₂), 2.40 (br s, 3H, CH₃). MS *m/z* 373 [M⁺+1].

15

Compound IN-053:

4-Hydroxyethyl-2-oxindole (5 g, 28.2 mmol) was dissolved in 20 mL of chlorosulfonic acid with stirring at room temperature. After 30 min, the reaction mixture was slowly added to ice water (300mL) with stirring. The solid was filtered and dried to afford 1.9g (28%) of 6,6-dioxo-3,6,8,9-tetrahydro-1*H*-7-oxa-6λ⁶-thia-3-aza-cyclopenta[α]naphthalen-2-one as a white powder.

20

¹H NMR (300 MHz, DMSO-*d*₆) δ 10.79 (s, 1H, NH), 7.65 (d, *J* = 8.2Hz, 1H, Ar-H), 6.90 (d, *J* = 8.2Hz, 1H, Ar-H), 4.87 (t, *J* = 5.7Hz, 2H, CH₂OSO₂), 3.51 (s, 2H, CH₂CO), 3.04 (t, *J* = 5.9Hz, 2H, CH₂Ar). MS *m/z* 239 [M⁺].

25

6,6-Dioxo-3,6,8,9-tetrahydro-1*H*-7-oxa-6λ⁶-thia-3-aza-cyclopenta[α]naphthalen-2-one (0.1 g, 0.4 mmol) was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde (1.2 eq., 0.08 g) to give 0.08 g (50%) of the desired product.

30 ¹H NMR (300 MHz, DMSO-*d*₆) δ 13.75 (s, 1H, NH), 11.55 (s, 1H, NH-CO), 7.95 (d, *J* = 2.9 Hz, 1H), 7.69 (d, *J* = 8.0 Hz, 1H), 7.55 (s, 1H, C=CH), 7.04 (d, *J* = 8.2 Hz), 4.95 (t, *J* = 5.3Hz, 2H, SO₃CH₂CH₂Ar), 4.5 (t, *J* = 5.8 Hz, 2H,

CO₂CH₂CH₂pyrrole), 3.55 (t, *J* = 5.0 Hz, 2H, SO₃CH₂CH₂Ar), 3.09 (t, *J* = 5.7 Hz, 2H, CO₂CH₂CH₂pyrrole).

Compound IN-054:

5 A mixture of 4-(2-hydroxy-ethyl)-1,3-dihydro-indol-2-one (35.4 mg, 0.2 mmol), 5-methyl-4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridine-1-carbaldehyde (35.6 mg, 0.2 mmol) and piperidine (0.1 mL) in ethanol (1 mL) was heated at 80 °C for 4 hours. The precipitate was collected by vacuum filtration, washed with ethanol and dried to give the title compound.

10 ¹HNMR (360 MHz, DMSO-*d*₆) δ 13.59 (br s, 1H, NH), 10.96 (br s, 1H, NH), 7.64 (d, *J* = 2.9 Hz, 1H), 7.54 (s, 1H, H-vinyl), 7.07 (t, *J* = 7.7 Hz, 1H), 6.83 (d, *J* = 7.7 Hz, 1H), 6.76 (d, *J* = 7.7 Hz, 1H), 4.86 (m, 1H, OH), 3.71 (m, 2H, CH₂OH), 3.57 (t, *J* = 6.9 Hz, 2H, CH₂), 3.07 (t, *J* = 6.9 Hz, 2H, CH₂), 2.98 (t, *J* = 6.6 Hz, 2H, CH₂), 2.95 (s, 3H, CH₃).

15 MS *m/z* 338 [M⁺+1].

Compound IN-055:

A mixture of 5-bromo-1,3-dihydro-indol-2-one (42.4 mg, 0.2 mmol), 5-methyl-4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridine-1-carbaldehyde (35.6 mg, 0.2 mmol) and piperidine (0.1 mL) in ethanol (1 mL) was heated at 80 °C for 4 hours. The precipitate was collected by vacuum filtration, washed with ethanol and dried to give the title compound.

25 ¹HNMR (360 MHz, DMSO-*d*₆) δ 13.47 (br s, 1H, NH), 11.04 (br s, 1H, NH), 8.06 (d, *J* = 1.9 Hz, 1H), 7.81 (s, 1H, H-vinyl), 7.68 (t, *J* = 3.3 Hz, 1H), 7.29 (dd, *J* = 1.9 & 8.5 Hz, 1H), 6.82 (d, *J* = 8.5 Hz, 1H), 3.58 (t, *J* = 6.7 Hz, 2H, CH₂), 3.08 (t, *J* = 6.7 Hz, 2H, CH₂), 2.95 (s, 3H, CH₃).

MS *m/z* 372/374 [M⁺+1].

Compound IN-056:

30 A mixture of 2-oxo-2,3-dihydro-1*H*-indole-5-carboxylic acid (35.4 mg, 0.2 mmol), 5-methyl-4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridine-1-

carbaldehyde (35.6 mg, 0.2 mmol) and piperidine (0.1 mL) in ethanol (1 mL) was heated at 80 °C for 4 hours. The reaction was treated with 1 N HCl and the precipitate was collected by vacuum filtration, washed with ethanol and dried to give the title compound.

5 ¹HNMR (360 MHz, DMSO-*d*₆) δ 13.46 (br s, 1H, NH), 12.6 (v br s, 1H, COOH), 11.26 (s, 1H, NH), 8.40 (s, 1H), 7.87 (s, 1H, H-vinyl), 7.80 (t, *J* = 8 Hz, 1H), 7.68 (dd, *J* = 3.1 Hz, 1H), 6.95 (d, *J* = 8 Hz, 1H), 3.57 (t, *J* = 6.6 Hz, 2H, CH₂), 3.13 (t, *J* = 6.6 Hz, 2H, CH₂), 2.95 (s, 3H, CH₃).

MS *m/z* 338 [M⁺+1].

10

Compound IN-057:

A mixture of 2-oxo-2,3-dihydro-1*H*-indole-5-sulfonic acid methylamide (45.2 mg, 0.2 mmol), 5-methyl-4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridine-1-carbaldehyde (35.6 mg, 0.2 mmol) and piperidine (0.1 mL) in ethanol (1 mL) was
15 heated at 80 °C for 4 hours. The precipitate was collected by vacuum filtration, washed with ethanol and dried to give the title compound.

¹HNMR (360 MHz, DMSO-*d*₆) δ 13.47 (br s, 1H, NH), 11.28 (v br s, 1H, NH), 8.24 (d, *J* = 1.5 Hz, 1H), 7.91 (s, 1H, H-vinyl), 7.71 (t, *J* = 3.2 Hz, 1H), 7.58 (dd, *J* = 1.5 & 8.2 Hz, 1H), 7.15 (m, 1H, CH₃NH), 7.05 (d, *J* = 8.2 Hz, 1H), 3.58 (t, *J* = 6.6 Hz, 2H, CH₂), 3.13 (t, *J* = 6.6 Hz, 2H, CH₂), 2.95 (s, 3H, CH₃), 2.42 (s, 3H, CH₃).

20

MS *m/z* 387 [M⁺+1].

Compound IN-058:

25 A mixture of 2-oxo-2,3-dihydro-1*H*-indole-5-sulfonic acid dimethylamide (48 mg, 0.2 mmol), 5-methyl-4-oxo-4,5,6,7-tetrahydro-2*H*-pyrrolo[3,4-*c*]pyridine-1-carbaldehyde (35.6 mg, 0.2 mmol) and piperidine (0.1 mL) in ethanol (1 mL) was heated at 80 °C for 4 hours. The precipitate was collected by vacuum filtration, washed with ethanol and dried to give the title compound.

30 ¹HNMR (360 MHz, DMSO-*d*₆) δ 13.48 (br s, 1H, NH), 11.35 (v br s, 1H, NH), 8.26 (d, *J* = 1.5 Hz, 1H), 8.0 (s, 1H, H-vinyl), 7.72 (t, *J* = 2.8 Hz, 1H), 7.54

(dd, $J = 1.5$ & 8.2 Hz, 1H), 7.08 (d, $J = 8.2$ Hz, 1H), 3.58 (t, $J = 6.6$ Hz, 2H, CH₂), 3.15 (t, $J = 6.6$ Hz, 2H, CH₂), 2.96 (s, 3H, CH₃), 2.61 (s, 6H, 2xCH₃).

MS m/z 401 [$M^+ + 1$].

5 Compound IN-059:

1,3-Dihydro-pyrrolo[2,3-*b*]pyridin-2-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS m/z 282 [$M^+ + 1$].

10 Compound IN-060:

4-(3-Chloro-4-fluoro-phenylamino)-5,7-dihydro-pyrrolo[2,3-*d*]pyrimidin-6-one was condensed with 4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-*c*]pyrrole-1-carbaldehyde to give the title compound.

MS m/z 426 [$M^+ + 1$].

15

ASSAY PROCEDURES

The following *in vitro* assays may be used to determine the level of activity and effect of the different compounds of the present invention on one or more of the
20 PKs. Similar assays can be designed along the same lines for any PK using techniques well known in the art.

Three general types of assays are useful for evaluating compounds: cellular/catalytic, cellular/biological and *in vivo*. The object of the cellular/catalytic assays is to determine the effect of a compound on the ability of a TK to
25 phosphorylate tyrosines on a known substrate in a cell. The object of the cellular/biological assays is to determine the effect of a compound on the biological response stimulated by a TK in a cell. The object of the *in vivo* assays is to determine the effect of a compound in an animal model of a particular disorder such as cancer.

30 The cellular/catalytic assays described herein are performed in an ELISA format. The general procedure is as follows: a compound is introduced to cells

expressing the test kinase, either naturally or recombinantly, for some period of time after which, if the test kinase is a receptor, a ligand known to activate the receptor is added. The cells are lysed and the lysate is transferred to the wells of an ELISA plate previously coated with a specific antibody recognizing the substrate of the enzymatic phosphorylation reaction. Non-substrate components of the cell lysate are washed away and the amount of phosphorylation on the substrate is detected with an antibody specifically recognizing phosphotyrosine compared with control cells that were not contacted with a test compound.

The cellular/biologic assays described herein measure the amount of DNA made in response to activation of a test kinase, which is a general measure of a proliferative response. The general procedure for this assay is as follows: a compound is introduced to cells expressing the test kinase, either naturally or recombinantly, for some period of time after which, if the test kinase is a receptor, a ligand known to activate the receptor is added. After incubation at least overnight, a DNA labeling reagent such as Bromodeoxy-uridine (BrdU) or 3H-thymidine is added. The amount of labeled DNA is detected with either an anti-BrdU antibody or by measuring radioactivity and is compared to control cells not contacted with a test compound.

Cellular/Catalytic Assays

Enzyme linked immunosorbent assays (ELISA) may be used to detect and measure the presence of PK activity. The ELISA may be conducted according to known protocols which are described in, for example, Voller, et al., 1980, "Enzyme-Linked Immunosorbent Assay," In: Manual of Clinical Immunology, 2d ed., edited by Rose and Friedman, pp 359-371 Am. Soc. Of Microbiology, Washington, D.C.

The disclosed protocol may be adapted for determining activity with respect to a specific PK. For example, the preferred protocols for conducting the ELISA experiments for specific PKs is provided below. Adaptation of these protocols for determining a compound's activity for other members of the RTK family, as well as for CTKs and STKs, is well within the scope of knowledge of those skilled in the art.

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5 The disclosed protocol may be adapted for determining activity with respect to a specific PK. For example, the preferred protocols for conducting the ELISA experiments for specific PKs is provided below. Adaptation of these protocols for determining a compound's activity for other members of the RTK family, as well as for CTKs and STKs, is well within the scope of knowledge of those skilled in the
10 art.

EXAMPLE 2: FLK-1

 An ELISA assay was conducted to measure the kinase activity of the FLK-1 receptor and more specifically, the inhibition or activation of TK activity on the FLK-1 receptor. Specifically, the following assay was conducted to measure kinase
15 activity of the FLK-1 receptor in cells genetically engineered to express Flk-1.

Materials and Methods.

Materials.

The following reagents and supplies were used:

- 20 a. Corning 96-well ELISA plates (Corning Catalog No. 25805-96);
- b. Cappel goat anti-rabbit IgG (catalog no. 55641);
- c. PBS (Gibco Catalog No. 450-1300EB);
- d. TBSW Buffer (50 mM Tris (pH 7.2), 150 mM NaCl and 0.1% Tween-20);
- e. Ethanolamine stock (10% ethanolamine (pH 7.0), stored at 4 °C);
- 25 f. HNTG buffer (20 mM HEPES buffer (pH 7.5), 150 mM NaCl, 0.2% Triton X-100, and 10% glycerol);
- g. EDTA (0.5 M (pH 7.0) as a 100X stock);
- h. Sodium orthovanadate (0.5 M as a 100X stock);
- i. Sodium pyrophosphate (0.2 M as a 100X stock);
- 30 j. NUNC 96 well V bottom polypropylene plates (Applied Scientific Catalog No. AS-72092);

- k. NIH3T3 C7#3 Cells (FLK-1 expressing cells);
- l. DMEM with 1X high glucose L-Glutamine (catalog No. 11965-050);
- m. FBS, Gibco (catalog no. 16000-028);
- n. L-glutamine, Gibco (catalog no. 25030-016);
- 5 o. VEGF, PeproTech, Inc. (catalog no. 100-20)(kept as 1 µg/100 µL stock in Milli-Q dH₂O and stored at -20 °C;
- p. Affinity purified anti-FLK-1 antiserum;
- q. UB40 monoclonal antibody specific for phosphotyrosine (*see*, Fendley, *et al.*, 1990, *Cancer Research* 50:1550-1558);
- 10 r. EIA grade Goat anti-mouse IgG-POD (BioRad catalog no. 172-1011);
- s. 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) solution (100 mM citric acid (anhydrous), 250 mM Na₂HPO₄ (pH 4.0), 0.5 mg/mL ABTS (Sigma catalog no. A-1888)), solution should be stored in dark at 4 °C until ready for use;
- t. H₂O₂ (30% solution) (Fisher catalog no. H325);
- 15 u. ABTS/H₂O₂ (15 mL ABTS solution, 2 µL H₂O₂) prepared 5 minutes before use and left at room temperature;
- v. 0.2 M HCl stock in H₂O;
- w. dimethylsulfoxide (100%) (Sigma Catalog No. D-8418); and
- y. Trypsin-EDTA (Gibco BRL Catalog No. 25200-049).

20

Protocol

The following protocol was used for conducting the assay:

- 1. Coat Corning 96-well ELISA plates with 1.0 µg per well Cappel Anti-rabbit IgG antibody in 0.1 M Na₂CO₃ pH 9.6. Bring final volume to 150 µL per well. Coat
25 plates overnight at 4 °C. Plates can be kept up to two weeks when stored at 4 °C.
- 2. Grow cells in Growth media (DMEM, supplemented with 2.0mM L-Glutamine, 10% FBS) in suitable culture dishes until confluent at 37 °C, 5% CO₂.
- 3. Harvest cells by trypsinization and seed in Corning 25850 polystyrene 96-well round bottom cell plates, 25,000 cells/well in 200 µL of growth media.
- 30 4. Grow cells at least one day at 37 °C, 5% CO₂.
- 5. Wash cells with D-PBS 1X.

6. Add 200 μ L/well of starvation media (DMEM, 2.0 mM l-Glutamine, 0.1% FBS). Incubate overnight at 37 °C, 5% CO₂.
7. Dilute Compounds 1:20 in polypropylene 96 well plates using starvation media. Dilute dimethylsulfoxide 1:20 for use in control wells.
- 5 8. Remove starvation media from 96 well cell culture plates and add 162 μ L of fresh starvation media to each well.
9. Add 18 μ L of 1:20 diluted Compound dilution (from step 7) to each well plus the 1:20 dimethylsulfoxide dilution to the control wells (+/- VEGF), for a final dilution of 1:200 after cell stimulation. Final dimethylsulfoxide is 0.5%. Incubate
- 10 the plate at 37 °C, 5% CO₂ for two hours.
10. Remove unbound antibody from ELISA plates by inverting plate to remove liquid. Wash 3 times with TBSW + 0.5% ethanolamine, pH 7.0. Pat the plate on a paper towel to remove excess liquid and bubbles.
11. Block plates with TBSW + 0.5% Ethanolamine, pH 7.0, 150 μ L per well.
- 15 Incubate plate thirty minutes while shaking on a microtiter plate shaker.
12. Wash plate 3 times as described in step 10.
13. Add 0.5 μ g/well affinity purified anti-FLU-1 polyclonal rabbit antiserum. Bring final volume to 150 μ L/well with TBSW + 0.5% ethanolamine pH 7.0. Incubate plate for thirty minutes while shaking.
- 20 14. Add 180 μ L starvation medium to the cells and stimulate cells with 20 μ L/well 10.0 mM sodium ortho vanadate and 500 ng/mL VEGF (resulting in a final concentration of 1.0 mM sodium ortho vanadate and 50 ng/mL VEGF per well) for eight minutes at 37 °C, 5% CO₂. Negative control wells receive only starvation medium.
- 25 15. After eight minutes, media should be removed from the cells and washed one time with 200 μ L/well PBS.
16. Lyse cells in 150 μ L/well HNTG while shaking at room temperature for five minutes. HNTG formulation includes sodium ortho vanadate, sodium pyrophosphate and EDTA.
- 30 17. Wash ELISA plate three times as described in step 10.
18. Transfer cell lysates from the cell plate to ELISA plate and incubate while

shaking for two hours. To transfer cell lysate pipette up and down while scrapping the wells.

19. Wash plate three times as described in step 10.
20. Incubate ELISA plate with 0.02 $\mu\text{g}/\text{well}$ UB40 in TBSW + 0.5% ethanolamine. Bring final volume to 150 $\mu\text{L}/\text{well}$. Incubate while shaking for 30 minutes.
21. Wash plate three times as described in step 10.
22. Incubate ELISA plate with 1:10,000 diluted EIA grade goat anti-mouse IgG conjugated horseradish peroxidase in TBSW + 0.5% ethanolamine, pH 7.0. Bring final volume to 150 $\mu\text{L}/\text{well}$. Incubate while shaking for thirty minutes.
23. Wash plate as described in step 10.
24. Add 100 μL of ABTS/ H_2O_2 solution to well. Incubate ten minutes while shaking.
25. Add 100 μL of 0.2 M HCl for 0.1 M HCl final to stop the color development reaction. Shake 1 minute at room temperature. Remove bubbles with slow stream of air and read the ELISA plate in an ELISA plate reader at 410 nm.

EXAMPLE 3: GST-FLK-1 BIOASSAY

This assay analyzes the tyrosine kinase activity of GST-Flk1 on poly glu tyr peptides.

Materials and Reagents:

1. Corning 96-well Elisa plates (Corning Catalog No. 25805-96).
2. poly glu tyr 4:1, lyophilizate (Sigma Catalog # P0275). Prepare 1 mg/mL poly glu tyr in sterile PBS and store in 1ml aliquots at -20 $^{\circ}\text{C}$.
3. Preparation of poly glu tyr (pEY) coated assay plates: Coat 2 $\mu\text{g}/\text{well}$ of poly glu tyr (pEY) in 100 μL PBS at room temperature for 2 hours or at +4 $^{\circ}\text{C}$ overnight. Cover plates well to prevent evaporation.
4. PBS Buffer: To make 1 liter of a 1x working solution, mix .02 g KH_2PO_4 , 1.15 g Na_2HPO_4 , 0.2 g KCl and 8 g NaCl in approx. 900 mL dH_2O . When all reagents have dissolved, adjust the pH to 7.2 with HCl. Bring total volume to 1 L

- with dH₂O.
5. PBS-Tw Buffer: To 1 L of PBS Buffer, add 1/0 mL Tween-20. Stir until dissolved.
6. TBB - Blocking Buffer: To make one liter of a 1x working solution, mix
5 1.21 g TRIS, 8.77 g NaCl, 1 mL TWEEN-20 in approximately 900 mL dH₂O.
Adjust pH to 7.2 with HCl. Add 10 g BSA, stir to dissolve. Bring total volume to 1 L with dH₂O. Filter to remove particulate matter.
7. 1% BSA in PBS: To make a 1x working solution, add 10 g BSA to approx.
990 mL PBS buffer, stir to dissolve. Adjust total volume to 1 L with PBS buffer,
10 filter to remove particulate matter.
8. 50 mM Hepes pH 7.5.
9. GST-FIk1cd purified from sf9 recombinant baculovirus transformation.
10. 4% DMSO in dH₂O.
11. 10 mM ATP in dH₂O.
- 15 12. 40 mM MnCl₂
13. Kinase Dilution Buffer (KDB): Mix 10 mL Hepes (pH 7.5), 1 mL of 5M NaCl, 40 µL of 100 mM NaVO₄ and 0.4 mL of 5% BSA in dH₂O with 88.56 mL of dH₂O.
14. NUNC 96-well V bottom polypropylene plates Applied Scientific Catalog #
20 AS-72092
15. EDTA: Mix 14.12 g ethylenediaminetetraacetic acid (EDTA) to approx. 70 mL dH₂O. Add 10 N NaOH until EDTA dissolves. Adjust pH to 8.0. Adjust total volume to 100 mL with dH₂O.
16. 1° Antibody Dilution Buffer: Mix 10 mL of 5% BSA in PBS buffer with
25 89.5 mL TBSTw.
17. Anti-phosphotyrosine monoclonal conjugated to horseradish peroxidase (PY99 HRP, Santa Cruz Biotech).
18. 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS, Moss, Cat. No. ABST).
- 30 19. 10% SDS.

PROCEDURE:

1. Coat Corning 96 well ELISA plates with 2 μ g of polyEY peptide in sterile PBS as described in step 3 of Materials and Reagents.
2. Remove unbound liquid from wells by inverting plate. Wash once with
5 TBSTw. Pat the plate on a paper towel to remove excess liquid.
3. Add 100 μ L of 1% BSA in PBS to each well. Incubate, with shaking, for 1 hr. at room temperature.
4. Repeat step 2.
5. Soak wells with 50 mM Hepes pH7.5 (150 μ L/well).
- 10 6. Dilute test compound with dH₂O/4% DMSO to 4 times the desired final assay concentration in 96-well polypropylene plates.
7. Add 25 μ L diluted test compound to ELISA plate. To control wells (wells which do not receive any test compound), add 25 μ L of dH₂O/4% DMSO.
8. Add 25 μ L of 40 mM MnCl₂ with 4x ATP (2 μ M) to all wells.
- 15 9. Add 25 μ L 0.5M EDTA to negative control wells.
10. Dilute GST-Flk1 0.005 μ g (5 ng)/well in KDB. For 50 ml KDB add 100 μ L of 0.050 mg/mL GST-Flk1 enzyme.
11. Add 50 μ L of diluted enzyme to each well.
12. Incubate, with shaking, for 15 minutes at room temperature.
- 20 13. Stop reaction by adding 50 μ L of 250 mM EDTA (pH 8.0).
14. Wash 3X with TBSTw and pat plate on paper towel to remove excess liquid.
15. Add 100 μ L per well anti-phosphotyrosine HRP conjugate, 1:5,000 dilution in antibody dilution buffer. Incubate, with shaking for 90 min. at room temperature.
16. Wash as described above in step 14.
- 25 17. Add 100 μ L of room temperature ABTS solution to each well.
18. Incubate, with shaking, for 10 to 15 minutes. Remove any bubbles.
19. Stop reaction by adding 20 μ L of 10% SDS.
20. Read assay on Dynatech MR7000 ELISA reader: test filter at 410 nM; reference filter at 630 nM.

EXAMPLE 4: PYK2 BIOASSAY

This assay is used to measure the in vitro kinase activity of HA epitope tagged full length pyk2 (FL.pyk2-HA) in an ELISA assay.

5 Materials and reagents:

1. Corning 96-well Elisa plates (Corning Catalog # 25805-96).
2. 12CA5 monoclonal anti-HA antibody
3. PBS (Dulbecco's Phosphate-Buffered Saline, Gibco Catalog # 450-1300EB)
4. TBST Buffer: Mix 8.766 g NaCl, 6.057 g TRIS and 1 ml of 0.1% Triton X-
10 100 in approx. 900 mL dH₂O. Adjust pH to 7.2, bring volume to 1 L.
5. Blocking Buffer: Mix 100 g of 10% BSA, 12.1 g of 100 mM TRIS, 58.44 g of 1 M NaCl and 10 mL of 1% TWEEN-20.
6. FL.pyk2-HA from sf9 cell lysates.
7. 4% DMSO in MilliQue H₂O.
- 15 8. 10 mM ATP in dH₂O.
9. 1 M MnCl₂.
10. 1 M MgCl₂.
11. 1 M Dithiothreitol (DTT).
12. 10X Kinase buffer phosphorylation mix: Mix 5.0 mL 1 M Hepes (pH 7.5),
20 0.2 mL 1 M MnCl₂, 1.0 mL MgCl₂, 1.0 mL 10% Triton X-100 in 2.8 ml dH₂O. Just prior to use, add 0.1 mL 1 M DTT.
13. NUNC 96-well V bottom polypropylene plates (Applied Scientific Catalog # AS-72092).
14. EDTA
- 25 15. Biotin conjugated anti-phosphotyrosine mab (Upstate Biotechnology Inc., clone 4G10 cat.# 16-103, ser. # 14495).
16. Vectastain Elite ABC reagent (Avidin peroxidase conjugate, Vector Laboratories (PK-6100).
17. ABTS Solution: Mix 19.21 g citric acid and 35.49 g Na₂HPO₄ in approx.
30 900 mL dH₂O. Adjust pH to 4.0 with phosphoric acid. Add 5 or 10 g ABST. When all dissolved, filter.

18. Hydrogen peroxide 30% solution.
19. ABTS/H₂O₂: Mix 15 mL ABTS solution with 3 µL 30% H₂O₂ 5 min. before use.
20. 0.2 M HCl.

5

Procedure:

1. Coat Corning 96 well ELISA plates with 0.5 µg per well 12CA5 anti-HA antibody in 100 µL PBS. Store overnight at 4 °c.
2. Remove unbound HA antibody from wells by inverting plate. Pat the plate
10 on a paper towel to remove excess liquid.
3. Add 150 µL Blocking Buffer to each well. Incubate, with shaking, for 1 hr at room temperature.
4. Wash plates with TBS-T.
5. Dilute lysate in PBS (1.5 µg lysate/100 µL PBS).
- 15 6. Add 100 µL of diluted lysate to each well. Shake at room temperature for 1 hr.
7. Wash as in step 4.
8. Add 50 µL of 2X kinase Buffer to ELISA plate containing captured pyk2-HA.
- 20 9. Add 25 µL of 40 µM test compound in 4% DMSO or 4% DMSO alone (control) to plate.
10. Add 25 µL of 0.5 M EDTA to negative control wells.
11. Add 25 µL of 20 µM ATP to all wells. Incubate, with shaking, for 10 minutes.
- 25 12. Stop reaction by adding 25 µL 500 mM EDTA (pH 8.0) to all wells.
13. Wash as in step 4.
14. Add 100 µL biotin conjugated anti-phosphotyrosine mab (1:5000 dilution in Blocking Buffer) to each well. Incubate, with shaking for 30 min. at room temperature.
- 30 15. Make up Vectastain ABC reagent. Allow 30 min. for complete coupling of the avidin with the biotinylated HRP. Add 1 drop (or 50 µL) reagent A to 15 mL

Blocking Buffer. Mix by inverting tube several times. Add 1 drop (or 50 μ L) reagent B and mix again. Allow ABC reagent to mix at room temperature while the biotin-4G10 anti-phosphotyrosine is incubating in the assay plate.

16. Wash as in step 4.
- 5 17. Add 100 μ L per well of prepared Vectastain peroxidase conjugate. Incubate, with shaking, for 30 min. at room temperature.
18. Wash as in step 4, then wash once with PBS.
19. Add 100 μ L of ABTS/ H_2O_2 solution to each well.
20. Incubate, with shaking, for 10 to 15 minutes. Remove any bubbles.
- 10 21. If necessary, stop reaction with the addition of 100 μ L of 0.2 M HCl per well.
22. Read assay on Dynatech MR7000 ELISA reader with test filter at 410 nM and reference filter at 630 nM.

15 EXAMPLE 5: FGFR1 BIOASSAY

This assay is used to measure the in vitro kinase activity of FGF1-R in an ELISA assay.

Materials and Reagents:

- 20 1. Costar 96-well Elisa plates (Corning Catalog # 3369).
2. Poly(Glu,Tyr) (Sigma Catalog # PO275).
3. PBS (Gibco Catalog # 450-1300EB)
4. 50 mM Hepes Buffer Solution.
5. Blocking Buffer (5% BSA/PBS).
- 25 6. Purified GST-FGFR1.
7. Kinase Dilution Buffer: Mix 500 μ L 1 M Hepes (GIBCO), 20 μ L 5% BSA/PBS, 10 μ L 100 mM sodium orthovanadate and 50 μ L 5 M NaCl.
8. 10 mM ATP
9. 1 M $MnCl_2$
- 30 10. ATP/ $MnCl_2$ phosphorylation mix: Mix 20 μ L ATP, 400 μ L $MnCl_2$ and 9.56 mL dH_2O .

11. NUNC 96-well V bottom polypropylene plates (Applied Scientific Catalog # AS-72092).
12. 0.5 M EDTA.
13. 0.05% TBST: Add 500 μ L TWEEN to 1 liter TBS.
- 5 14. Rabbit polyclonal anti-phosphotyrosine serum.
15. Goat anti-rabbit IgG peroxidase conjugate (Biosource, Catalog # ALI0404).
16. ABTS Solution
17. 30% Hydrogen peroxide.
18. ABTS/H₂O₂

10

Procedure:

1. Coat Costar 96 well ELISA plates with 1 μ g per well Poly(Glu,Tyr) in 100 μ L PBS. Store overnight at 4 °C.
2. Wash coated plates once with PBS.
- 15 3. Add 150 μ L of 5%BSA/PBS Blocking Buffer to each well. Incubate, with shaking, for 1 hr.room temperature.
4. Wash plate 2x with PBS, then once with 50 mM Hepes. Pat plates on a paper towel to remove excess liquid and bubbles.
5. Add 25 μ L of 0.4 mM test compound in 4% DMSO or 4% DMSO alone
- 20 (controls) to plate.
6. Dilute purified GST-FGFR1 in Kinase Dilution Buffer (5 ng kinase/50 μ L KDB/well).
7. Add 50 μ L of diluted kinase to each well.
8. Start kinase reaction by adding 25 μ L /well of freshly prepared ATP/Mn⁺⁺
- 25 (0.4 mL 1 M MnCl₂, 40 μ L 10 mM ATP, 9.56 mL dH₂O), freshly prepared).
9. This is a fast kinase reaction and must be stopped with 25 μ L of 0.5M EDTA in a manner similar to the addition of ATP.
10. Wash plate 4x with fresh TBST.
11. Make up Antibody Dilution Buffer: Per 50 mL: Mix 5 ml of 5% BSA, 250
- 30 μ L of 5% milk and 50 μ L of 100mM sodium vanadate, bring to final volume with 0.05% TBST.

12. Add 100 μ l per well of anti-phosphotyrosine (1:10000 dilution in ADB).
Incubate, with shaking for 1 hr. at room temperature.
13. Wash as in step 10.
14. Add 100 μ L per well of Biosource Goat anti-rabbit IgG peroxidase conjugate
5 (1:6000 dilution in ADB). Incubate, with shaking for 1 hr. at room temperature.
15. Wash as in step 10 and then with PBS to remove bubbles and excess
TWEEN.
16. Add 100 μ L of ABTS/H₂O₂ solution to each well.
17. Incubate, with shaking, for 10 to 20 minutes. Remove any bubbles.
- 10 18. Read assay on Dynatech MR7000 ELISA reader: test filter at 410 nM,
reference filter at 630 nM.

EXAMPLE 6: CELLULAR HER-2 KINASE ASSAY

- This assay is used to measure HER-2 kinase activity in whole cells in an
15 ELISA format.

Materials and Reagents:

1. DMEM (GIBCO Catalog #11965-092).
2. Fetal Bovine Serum (FBS, GIBCO Catalog #16000-044), heat inactivated in
20 a water bath for 30 min. at 56 °C.
3. Trypsin (GIBCO Catalog #25200-056).
4. L-Glutamine (GIBCO Catalog #25030-081)
5. HEPES (GIBCO Catalog #15630-080).
6. Growth Media: Mix 500 mL DMEM, 55 mL heat inactivated FBS, 10 mL
25 HEPES and 5.5 mL L-Glutamine.
7. Starve Media: Mix 500 mL DMEM, 2.5 mL heat inactivated FBS, 10 mL
HEPES and 5.5 mL L-Glutamine.
8. PBS.
9. Flat Bottom 96-well Tissue Culture Micro Titer Plates (Corning Catalog #
30 25860).
10. 15 cm Tissue Culture Dishes (Corning Catalog #08757148).

11. Corning 96-well ELISA Plates.
12. NUNC 96-well V bottom polypropylene plates.
13. Costar Transfer Cartidges for the Transtar 96 (Costar Catalog #7610).
14. SUMO 1: monoclonal anti-EGFR antibody.
- 5 15. TBST Buffer
16. Blocking Buffer : 5% Carnation Instant Milk® in PBS.
17. EGF Ligand: EGF-201, Shinko American, Japan. Suspend powder in 100 µL of 10 mM HCl. Add 100 µL 10mM NaOH. Add 800 µL PBS and transfer to an Eppendorf tube for storage at -20 °C.
- 10 18. HNTG Lysis Buffer:
For Stock 5X HNTG: Mix 23.83 g Hepes, 43.83 g NaCl, 500 mL glycerol and 100 mL Triton X-100 and enough dH₂O to make 1 L of total solution.
For 1X HNTG*: Mix 2 mL HNTG, 100 µL 0.1 M Na₃VO₄, 250 µL 0.2M Na₄P₂O₇, and 100 µL EDTA.
- 15 19. EDTA
20. Na₃VO₄:
To make stock solution: Mix 1.84 g Na₃VO₄ with 90 mL dH₂O. Adjust pH to 10. Boil in microwave for one minute (solution becomes clear). Cool to room temperature. Adjust pH to 10. Repeat heating/cooling cycle until pH remains at 10.
- 20 21. 200 mM Na₄P₂O₇.
22. Rabbit polyclonal antiserum specific for phosphotyrosine (anti-Ptyr antibody).
23. Affinity purified antiserum, goat anti-rabbit IgG antibody, peroxidase conjugate (Biosource Cat # ALI0404).
- 25 24. ABTS Solution
25. 30 % Hydrogen peroxide solution.
26. ABTS/H₂O₂
27. 0.2 M HCl

30 Procedure:

1. Coat Corning 96 well ELISA plates with SUMO1 at 1.0 µg per well in PBS,

100 μ L final volume/well. Store overnight at 4 °C.

2. On day of use, remove coating buffer and wash plate 3 times with dH₂O and once with TBST buffer. All washes in this assay should be done in this manner, unless otherwise specified.
- 5 3. Add 100 μ L of Blocking Buffer to each well. Incubate plate, with shaking, for 30 min. at room temperature. Just prior to use, wash plate as described above.
4. Use EGFr/HER-2 chimera/3T3-C7 cell line for this assay.
5. Choose dishes having 80-90 % confluence. Collect cells by trypsinization and centrifuge at 1000 rpm at room temperature for 5 min.
- 10 6. Resuspend cells in starve medium and count with trypan blue. Viability above 90 % is required. Seed cells in starve medium at a density of 2,500 cells per well, 90 μ L per well, in a 96 well microtiter plate. Incubate seeded cells overnight at 37 °C under 5% CO₂.
7. Start the assay two days after seeding.
- 15 8. Test compound dilution:

Primary screening :

Samples are diluted directly into a polypropylene plate containing starve-DMEM. This dilution will be 1:10 or greater, depending on the samples being screened. The same amount of DMSO is put into the control wells. All wells are
20 then transferred to the cell plate at a 1:10 dilution (10 μ L of sample and media into 90 μ L of starve media). The final DMSO concentration will be 1% or lower.

Secondary screening :

Ten samples are put into wells 2-11 of row A of a polypropylene plate. These wells contain straight starve-DMEM. For a 1:10 dilution, use 10 μ L of test
25 compound solution in 90 μ L of media. The rest of the wells (including control) will have a DMSO/media mixture. The percentage of DMSO in this mixture is determined by the first dilution factor, e.g., in this example, 1:10. The DMSO concentration is therefore 10%. An equal amount of drug and media from row A is put into row B, containing DMSO and media. The same amount is then taken out
30 and put into row C, etc. These are 1:2 dilutions. All wells are then transferred to the cell plate at 1:10 dilution (10 μ L of sample and media into 90 μ L of starve media).

The final DMSO concentration will be 1% or lower.

9. Incubate under 5% CO₂ at 37 °C for 2 hours.
10. Prepare EGF ligand by diluting stock EGF (16.5 µM) in warm DMEM to 150 nM.
- 5 11. Prepare fresh HNTG* sufficient for 100 µL per well; place on ice.
12. After 2 hour incubation with test compound, add prepared EGF ligand to cells, 50 ul per well, for a final concentration of 50 nM. Positive control wells receive the same amount of EGF. Negative controls do not receive EGF. Incubate at 37 °C for 10 min.
- 10 13. Remove test compound, EGF, and DMEM. Wash cells once with PBS.
14. Transfer HNTG* to cells, 100 µL per well. Place on ice for 5 minutes. Meanwhile, remove blocking buffer from ELISA plate and wash.
15. With a pipette tip securely fitted to a micropipettor, scrape cells from plate and homogenize cell material by repeatedly aspirating and dispensing the HNTG* lysis buffer. Transfer lysate to a coated, blocked, washed ELISA plate.
- 15 Alternatively, one may use a Costar transfer cartridge to transfer lysate to the ELISA plate.
16. Incubate, with shaking, at room temperature for one hr.
17. Remove lysate, wash. Transfer freshly diluted anti-Ptyr antibody (1:3000 in 20 TBST) to ELISA plate, 100 µL per well.
18. Incubate, with shaking, at room temperature, for 30 min.
19. Remove anti-Ptyr antibody, wash. Transfer freshly diluted BIOSOURCE antibody to ELISA plate(1:8000 in TBST, 100 µL per well).
20. Incubate, with shaking, at room temperature for 30 min.
- 25 21. Remove BIOSOURCE antibody, wash. Transfer freshly prepared ABTS/H₂O₂ solution to ELISA plate, 100 µL per well.
22. Incubate, with shaking, for 5-10 minutes. Remove any bubbles.
23. If necessary, stop reaction with the addition of µL of 0.2 M HCl per well.
24. Read assay on Dynatech MR7000 ELISA reader: test filter set at 410 nM;
- 30 reference filter at 630 nM.

EXAMPLE 7: CDK2/CYCLIN A ASSAY

The following protocol describes the procedures used to analyze protein serine/threonine kinase activity of cdk2/cyclin A in an SPA. The procedure also describes the protocol for the initial screening of drugs for inhibition or activation of the kinase activity.

Materials and Reagents:

1. Wallac 96-well polyethylene terephthalate (flexi) plates (Wallac Catalog # 1450-401).
- 10 2. Amersham Redivue [$\gamma^{33}\text{P}$] ATP (Amersham catalog #AH 9968).
3. Amersham streptavidin coated polyvinyltoluene SPA beads (Amersham catalog #RPNQ0007). Reconstitute beads in PBS without magnesium or calcium, at 20 mg/mL. Store reconstituted beads at 4 °C.
4. Activated cdk2/cyclin A enzyme complex purified from Sf9 cells, -80 °C, 15 200 μL aliquots
5. Biotinylated peptide substrate (deb-tide). Peptide biotin-X-PKTPKKAKKL dissolved in dH_2O at a concentration of 5 mg/mL. Stored at -80 °C in 100 μL aliquots.
6. Peptide/ATP Mixture:

20

Reagent	Stock Concentration	2.5 X Working Concentration	Amount per 10 mL	Final Well Concentration
dH_2O		10 mL	9.979 mL	---
Cold ATP	10 mM	1.25 μM	0.00125 mL	0.5 μM
Debtide	5 mg/mL	0.005 mg/mL	0.010 mL	0.1 $\mu\text{g/well}$
$\gamma^{33}\text{P}$ ATP	10 $\mu\text{Ci}/\mu\text{L}$	10 $\mu\text{Ci}/\text{mL}$	0.010 mL	0.2 $\mu\text{Ci/well}$

7. 2.5 X kinase buffer

Reagent	Stock solution	Amount per 10 mL	Working Concentration	Final Well Concentration
dH ₂ O	55.5 M	8.85 mL		-----
Tris pH7.4	1 M	0.625 mL	62.5 mM	25 mM TRIS
MgCl ₂	1 M	0.25 mL	25 mM	10 mM MgCl ₂
NP40	10%	0.25 mL	0.25%	0.1 % NP40
*DTT add fresh	1 M	0.025 mL	2.5 mM	1 mM DTT

8. 10 mM ATP (Sigma Catalog # A-5394).

9. 1 M Tris, pH 7.4

5 10. 1 M MgCl₂

11. 1 M DTT

12. PBS (Dulbecco's Phosphate-Buffered Saline) without magnesium or calcium
(Gibco Catalog # 14190-144)

13. EDTA (14.12 g per 100 mL).

10 14. Stop solution:

Reagent	Stock solution	Amount per 10 mL	Working Concentration
PBS		9.25 mL	
ATP	100 mM	0.005 mL	50 uM
EDTA	0.5 M	0.1 mL	5 mM
Triton X-100	10%	0.1 mL	0.1%
SPA beads	20 mg/mL	1.25 mL	0.5 mg/well (200 µL)

Procedure:1. Prepare solutions of inhibitors at 5x the desired final concentration in 5%
DMSO. Add 10 µL to each well. For negative controls, add 10 µL 5%

15 DMSO.

2. Dilute 5 µL of cdk2/cyclin A solution into 2.1 mL 2x kinase buffer (per

plate).

3. Add 20 μ L enzyme per well. This can be added using a hand pipette or by using the Titertek Multidrop.
4. Add 10 μ L of 0.5 M EDTA to the neagtive control wells.
- 5 5. To start kinase reaction, add 20 μ L of peptide/ATP mixture using either a hand pipette or the Titertek Multidrop. Let sit on benchtop behind reactive shield for 1 hr.
6. Add 200 μ L stop solution per well using either the Titertek Multidrop or hand pipette.
- 10 7. Let stand at least 10 min.
8. Spin plate approx. 2300 rpm 3-5 min.
9. Count plate on Trilux reader using protocol #28 (Brian's SPA assay).

EXAMPLE 8: PDGF-R ELISA

- 15 All cell culture media, glutamine, and fetal bovine serum were purchased from Gibco Life Technologies (Grand Island, NY) unless otherwise specified. All cells were grown in a humid atmosphere of 90-95% air and 5-10% CO₂ at 37 °C. All cell lines were routinely subcultured twice a week and were negative for mycoplasma as determined by the Mycotect method (Gibco).
- 20 For ELISA assays, cells (U1242, obtained from Joseph Schlessinger, NYU) were grown to 80-90% confluency in growth medium (MEM with 10% FBS, NEAA, 1 mM NaPyr and 2 mM GLN) and seeded in 96-well tissue culture plates in 0.5% serum at 25,000 to 30,000 cells per well. After overnight incubation in 0.5% serum-containing medium, cells were changed to serum-free medium and treated
- 25 with test compound for 2 hr in a 5% CO₂, 37 °C incubator. Cells were then stimulated with ligand for 5-10 minute followed by lysis with HNTG (20 mM Hepes, 150 mM NaCl, 10% glycerol, 5 mM EDTA, 5 mM Na₃VO₄, 0.2% Triton X-100, and 2 mM NaPyr). Cell lysates (0.5 mg/well in PBS) were transferred to ELISA plates previously coated with receptor-specific antibody and which had been
- 30 blocked with 5% milk in TBST (50 mM Tris-HCl pH 7.2, 150 mM NaCl and 0.1% Triton X-100) at room temperature for 30 min. Lysates were incubated with shaking

for 1 hour at room temperature. The plates were washed with TBST four times and then incubated with polyclonal anti-phosphotyrosine antibody at room temperature for 30 minutes. Excess anti-phosphotyrosine antibody was removed by rinsing the plate with TBST four times. Goat anti-rabbit IgG antibody was added to the ELISA
 5 plate for 30 min at room temperature followed by rinsing with TBST four more times. ABTS (100 mM citric acid, 250 mM Na_2HPO_4 and 0.5 mg/mL 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) plus H_2O_2 (1.2 mL 30% H_2O_2 to 10 mL ABTS) was added to the ELISA plates to start color development. Absorbance at 410 nm with a reference wavelength of 630 nm was recorded about 15 to 30 min
 10 after ABTS addition.

EXAMPLE 9: IGF-I RECEPTOR ELISA

The following protocol may be used to measure phosphotyrosine level on IGF-I receptor, which indicates IGF-I receptor tyrosine kinase activity.

15

Materials and Reagents

The following materials and reagents were used:

- a. The cell line used in this assay is 3T3/IGF-1R, a cell line genetically engineered to overexpresses IGF-1 receptor.
- 20 b. NIH3T3/IGF-1R is grown in an incubator with 5% CO_2 at 37 °C. The growth media is DMEM + 10% FBS (heat inactivated)+ 2 mM L-glutamine.
- c. Affinity purified anti-IGF-1R antibody 17-69.
- d. D-PBS:

KH_2PO_4	0.20 g/L
K_2HPO_4	2.16 g/L
KCl	0.20 g/L
NaCl	8.00 g/L(pH 7.2)
- e. Blocking Buffer: TBST plus 5% Milk (Carnation Instant Non-Fat Dry Milk).
- 30 f. TBST buffer:

Tris-HCl	50 mM
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NaCl 150 mM (pH 7.2/HCl 10 N)

Triton X-100 0.1%

Stock solution of TBS (10X) is prepared, and Triton X-100 is added to the buffer during dilution.

5

g. HNTG buffer:

HEPES 20 mM

NaCl 150 mM (pH 7.2/HCl 1N)

Glycerol 10%

10 Triton X-100 0.2%

Stock solution (5X) is prepared and kept at 4 °C.

h. EDTA/HCl: 0.5 M pH 7.0 (NaOH) as 100X stock.

i. Na_3VO_4 : 0.5 M as 100X stock and aliquots are kept in -80 °C.

15 j. $\text{Na}_4\text{P}_2\text{O}_7$: 0.2 M as 100X stock.

k. Insulin-like growth factor-1 from Promega (Cat# G5111).

l. Rabbit polyclonal anti-phosphotyrosine antiserum.

m. Goat anti-rabbit IgG, POD conjugate (detection antibody), Tago (Cat. No. 4520, Lot No. 1802): Tago, Inc., Burlingame, CA.

20 n. ABTS (2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)) solution:

Citric acid 100 mM

Na_2HPO_4 250 mM (pH 4.0/1 N HCl)

ABTS 0.5 mg/mL

ABTS solution should be kept in dark and 4°C. The solution should be discarded

25 when it turns green.

o. Hydrogen Peroxide: 30% solution is kept in the dark and at 4 °C.

Procedure

30 All the following steps are conducted at room temperature unless it is specifically indicated. All ELISA plate washings are performed by rinsing the plate with tap

water three times, followed by one TBST rinse. Pat plate dry with paper towels.

A. Cell Seeding:

1. The cells, grown in tissue culture dish (Corning 25020-100) to 80-90% confluence, are harvested with Trypsin-EDTA (0.25%, 0.5 mL/D-100, GIBCO).
- 5 2. Resuspend the cells in fresh DMEM + 10% FBS + 2 mM L-Glutamine, and transfer to 96-well tissue culture plate (Corning, 25806-96) at 20,000 cells/well (100 μ L/well). Incubate for 1 day then replace medium to serum-free medium (90/ μ L) and incubate in 5% CO₂ and 37 °C overnight.

B. ELISA Plate Coating and Blocking:

- 10 1. Coat the ELISA plate (Corning 25805-96) with Anti-IGF-1R Antibody at 0.5 μ g/well in 100 μ L PBS at least 2 hours.
2. Remove the coating solution, and replace with 100 μ L Blocking Buffer, and shake for 30 minutes. Remove the blocking buffer and wash the plate just before adding lysate.

15 C. Assay Procedures:

1. The drugs are tested in serum-free condition.
2. Dilute drug stock (in 100% DMSO) 1:10 with DMEM in 96-well polypropylene plate, and transfer 10 μ L/well of this solution to the cells to achieve final drug dilution 1:100, and final DMSO concentration of 1.0%. Incubate the cells in
- 20 5% CO₂ at 37 °C for 2 hours.

3. Prepare fresh cell lysis buffer (HNTG*)

	HNTG	2 mL
	EDTA	0.1 mL
	Na ₃ VO ₄	0.1 mL
25	Na ₄ (P ₂ O ₇)	0.1 mL
	H ₂ O	7.3 mL

4. After drug incubation for two hours, transfer 10 μ L/well of 200nM IGF-1 Ligand in PBS to the cells (Final Conc. = 20 nM), and incubate at 5% CO₂ at 37 °C for 10 minutes.
- 30 5. Remove media and add 100 μ L/well HNTG* and shake for 10 minutes. Look at cells under microscope to see if they are adequately lysed.

6. Use a 12-channel pipette to scrape the cells from the plate, and homogenize the lysate by repeated aspiration and dispensing. Transfer all the lysate to the antibody coated ELISA plate, and shake for 1 hour.
7. Remove the lysate, wash the plate, transfer anti-pTyr (1:3,000 with TBST) 100 μ L/well, and shake for 30 minutes.
8. Remove anti-pTyr, wash the plate, transfer TAGO (1:3,000 with TBST) 100 μ L/well, and shake for 30 minutes.
9. Remove detection antibody, wash the plate, and transfer fresh ABTS/H₂O₂ (1.2 μ L H₂O₂ to 10 ml ABTS) 100 μ L/well to the plate to start color development.
10. Measure OD at 410 nm with a reference wavelength of 630 nm in Dynatec MR5000.

EXAMPLE 10: EGF Receptor ELISA

- EGF Receptor kinase activity in cells genetically engineered to express human EGF-R was measured as described below:

Materials and Reagents

The following materials and reagents were used:

- a. EGF Ligand: stock concentration = 16.5 μ M; EGF 201, TOYOBO, Co., Ltd. Japan.
- b. 05-101 (UBI) (a monoclonal antibody recognizing an EGFR extracellular domain).
- c. Anti-phosphotyrosine antibody (anti-Ptyr) (polyclonal).
- d. Detection antibody: Goat anti-rabbit IgG horse radish peroxidase conjugate, TAGO, Inc., Burlingame, CA.
- e. TBST buffer:

Tris-HCl, pH 7	50 mM
NaCl	150 mM
Triton X-100	0.1
- f. HNTG 5X stock:

HEPES	0.1 M
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NaCl	0.75 M
Glycerol	50
Triton X-100	1.0%

g. ABTS stock:

5	Citric Acid	100 mM
	Na ₂ HPO ₄	250 mM
	HCl, conc.	4.0 pH
	ABTS*	0.5 mg/mL

Keep solution in dark at 4 °C until used.

10 h. Stock reagents of:

EDTA 100 mM pH 7.0
Na₃VO₄ 0.5 M
Na₄(P₂O₇) 0.2 M

15 Procedure

The following protocol was used:

A. Pre-coat ELISA Plate

1. Coat ELISA plates (Corning, 96 well, Cat. #25805-96) with 05-101 antibody
20 at 0.5 µg per well in PBS, 150 µL final volume/well, and store overnight at 4 °C.
Coated plates are good for up to 10 days when stored at 4 °C.
2. On day of use, remove coating buffer and replace with blocking buffer (5% Carnation Instant Non-Fat Dry Milk in PBS). Incubate the plate, shaking, at room temperature (about 23 °C to 25 °C) for 30 minutes. Just prior to use, remove
25 blocking buffer and wash plate 4 times with TBST buffer.

B. Seeding Cells

1. NIH 3T3/C7 cell line (Honegger, et al., Cell 51:199-209, 1987) can be use
for this assay.
2. Choose dishes having 80-90% confluence for the experiment. Trypsinize
30 cells and stop reaction by adding 10% CS DMEM medium. Suspend cells in DMEM
medium (10% CS DMEM medium) and centrifuge once at 1000 rpm at room

temperature for 5 minutes.

3. Resuspend cells in seeding medium (DMEM, 0.5% bovine serum), and count the cells using trypan blue. Viability above 90% is acceptable. Seed cells in DMEM medium (0.5% bovine serum) at a density of 10,000 cells per well, 100 μ L per well,
5 in a 96 well microtiter plate. Incubate seeded cells in 5% CO₂ at 37 °C for about 40 hours.

C. Assay Procedures.

1. Check seeded cells for contamination using an inverted microscope. Dilute drug stock (10 mg/ml in DMSO) 1:10 in DMEM medium, then transfer 5 μ L to a
10 test well for a final drug dilution of 1:200 and a final DMSO concentration of 1%. Control wells receive DMSO alone. Incubate in 5% CO₂ at 37 °C for one hour.
2. Prepare EGF ligand: dilute stock EGF in DMEM so that upon transfer of 10 μ L dilute EGF (1:12 dilution), 25 nM final concentration is attained.
3. Prepare fresh 10 ml HNTG* sufficient for 100 μ L per well wherein HNTG*
15 comprises: HNTG stock (2.0 mL), milli-Q H₂O (7.3 mL), EDTA, 100 mM, pH 7.0 (0.5 mL), Na₃VO₄ 0.5 M (0.1 mL) and Na₄(P₂O₇), 0.2 M (0.1 mL).
4. Place on ice.
5. After two hours incubation with drug, add prepared EGF ligand to cells, 10 μ L per well, to yield a final concentration of 25 nM. Control wells receive DMEM
20 alone. Incubate, shaking, at room temperature, for 5 minutes.
6. Remove drug, EGF, and DMEM. Wash cells twice with PBS. Transfer HNTG* to cells, 100 μ L per well. Place on ice for 5 minutes. Meanwhile, remove blocking buffer from other ELISA plate and wash with TBST as described above.
7. With a pipette tip securely fitted to a micropipettor, scrape cells from plate
25 and homogenize cell material by repeatedly aspirating and dispensing the HNTG* lysis buffer. Transfer lysate to a coated, blocked, and washed ELISA plate. Incubate shaking at room temperature for one hour.
8. Remove lysate and wash 4 times with TBST. Transfer freshly diluted anti-Ptyr antibody to ELISA plate at 100 μ L per well. Incubate shaking at room
30 temperature for 30 minutes in the presence of the anti-Ptyr antiserum (1:3000 dilution in TBST).

9. Remove the anti-Ptyr antibody and wash 4 times with TBST. Transfer the freshly diluted TAGO 30 anti-rabbit IgG antibody to the ELISA plate at 100 μ L per well. Incubate shaking at room temperature for 30 minutes (anti-rabbit IgG antibody: 1:3000 dilution in TBST).
- 5 10. Remove detection antibody and wash 4 times with TBST. Transfer freshly prepared ABTS/H₂O₂ solution to ELISA plate, 100 μ L per well. Incubate at room temperature for 20 minutes. ABTS/H₂O₂ solution: 1.2 μ L 30% H₂O₂ in 10 mL ABTS stock.
11. Stop reaction by adding 50 μ L 5N H₂SO₄ (optional), and determine O.D. at
10 410 nm.
12. The maximal phosphotyrosine signal is determined by subtracting the value of the negative controls from the positive controls. The percent inhibition of phosphotyrosine content for extract-containing wells is then calculated, after subtraction of the negative controls.

15

EXAMPLE 11: Met Autophosphorylation Assay - ELISA

This assay determines Met tyrosine kinase activity by analyzing Met protein tyrosine kinase levels on the Met receptor.

20 Materials and Reagents

The following materials and reagents were used:

- a. HNTG (5X stock solution): Dissolve 23.83 g HEPES and 43.83 g NaCl in about 350 mL dH₂O. Adjust pH to 7.2 with HCl or NaOH, add 500 mL glycerol and 10 mL Triton X-100, mix, add dH₂O to 1 L total volume. To make 1 L of 1X
25 working solution add 200 mL 5X stock solution to 800 mL dH₂O, check and adjust pH as necessary, store at 4 °C.
- b. PBS (Dulbecco's Phosphate-Buffered Saline), Gibco Cat. # 450-1300EB (1X solution).
- c. Blocking Buffer: in 500 ml dH₂O place 100 g BSA, 12.1 g Tris-pH7.5,
30 58.44 g NaCl and 10 mL Tween-20, dilute to 1 L total volume.
- d. Kinase Buffer: To 500 mL dH₂O add 12.1 g TRIS pH7.2, 58.4 g NaCl, 40.7

- g MgCl_2 and 1.9 g EGTA; bring to 1 L total volume with dH_2O .
- e. PMSF (Phenylmethylsulfonyl fluoride), Sigma Cat. # P-7626, to 435.5 mg, add 100% ethanol to 25 mL total volume, vortex.
- f. ATP (Bacterial Source), Sigma Cat. # A-7699, store powder at -20°C ; to
5 make up solution for use, dissolve 3.31 mg in 1 mL dH_2O .
- g. RC-20H HRPO Conjugated Anti-Phosphotyrosine, Transduction Laboratories Cat. # E120H.
- h. Pierce 1-Step (TM) Turbo TMB-ELISA (3,3',5,5'-tetramethylbenzidine, Pierce Cat. # 34022.
- 10 i. H_2SO_4 , add 1 mL conc. (18 N) to 35 mL dH_2O .
- j. TRIS HCL, Fischer Cat. # BP152-5; to 121.14 g of material, add 600 mL MilliQ H_2O , adjust pH to 7.5 (or 7.2) with HCl, bring volume to 1 L with MilliQ H_2O .
- k. NaCl, Fischer Cat. # S271-10, make up 5 M solution.
- 15 l. Tween-20, Fischer Cat. # S337-500.
- m. Na_3VO_4 , Fischer Cat. # S454-50, to 1.8 g material add 80 ml MilliQ H_2O , adjust pH to 10.0 with HCl or NaOH, boil in microwave, cool, check pH, repeat procedure until pH stable at 10.0, add MilliQ H_2O to 100 ml total volume, make 1 mL aliquots and store at -80°C .
- 20 n. MgCl_2 , Fischer Cat. # M33-500, make up 1 M solution.
- o. HEPES, Fischer Cat. # BP310-500, to 200 ml MilliQ H_2O , add 59.6 g material, adjust pH to 7.5, bring volume to 250 mL total, sterile filter.
- p. Albumin, Bovine (BSA), Sigma Cat. # A-4503, to 30 grams material add sterile distilled water to make total volume of 300 mL, store at 4°C .
- 25 q. TBST Buffer: to approx. 900 mL dH_2O in a 1 L graduated cylinder add 6.057 g TRIS and 8.766 g NaCl, when dissolved, adjust pH to 7.2 with HCl, add 1.0 mL Triton X-100 and bring to 1 L total volume with dH_2O .
- r. Goat Affinity purified antibody Rabbit IgG (whole molecule), Cappel Cat. # 55641.
- 30 s. Anti h-Met (C-28) rabbit polyclonal IgG antibody, Santa Cruz Chemical Cat. # SC-161.

- t. Transiently Transfected EGFR/Met chimeric cells (EMR) (Komada, et al., Oncogene, 8:2381-2390 (1993).
- u. Sodium Carbonate Buffer, (Na_2CO_3 , Fischer Cat. # S495): to 10.6 g material add 800 mL MilliQ H_2O , when dissolved adjust pH to 9.6 with NaOH, bring up to 1 L
- 5 total volume with MilliQ H_2O , filter, store at 4 °C.

Procedure

- All of the following steps are conducted at room temperature unless it is specifically indicated otherwise. All ELISA plate washing is by rinsing 4X with
- 10 TBST.

A. EMR Lysis

This procedure can be performed the night before or immediately prior to the start of receptor capture.

- 15 1. Quick thaw lysates in a 37 °C waterbath with a swirling motion until the last crystals disappear.
- 2. Lyse cell pellet with 1X HNTG containing 1 mM PMSF. Use 3 ml of HNTG per 15 cm dish of cells. Add ½ the calculated HNTG volume, vortex the tube for 1 min., add the remaining amount of HNTG, vortex for another min.
- 20 3. Balance tubes, centrifuge at 10,000x g for 10 min at 4 °C.
- 4. Pool supernatants, remove an aliquot for protein determination.
- 5. Quick freeze pooled sample in dry ice/ethanol bath. This step is performed regardless of whether lysate will be stored overnight or used immediately following protein determination.
- 25 6. Perform protein determination using standard bicinchoninic acid (BCA) method (BCA Assay Reagent Kit from Pierce Chemical Cat. # 23225).

B. ELISA Procedure

- 1. Coat Corning 96 well ELISA plates with 5 µg per well Goat anti-Rabbit
- 30 antibody in Carbonate Buffer for a total well volume of 50 µL. Store overnight at 4 °C.

2. Remove unbound Goat anti-rabbit antibody by inverting plate to remove liquid.
3. Add 150 μ L of Blocking Buffer to each well. Incubate for 30 min. at room temperature with shaking.
- 5 4. Wash 4X with TBST. Pat plate on a paper towel to remove excess liquid and bubbles.
5. Add 1 μ g per well of Rabbit anti-Met antibody diluted in TBST for a total well volume of 100 μ L.
6. Dilute lysate in HNTG (90 μ g lysate/100 μ L)
- 10 7. Add 100 μ L of diluted lysate to each well. Shake at room temperature for 60 min.
8. Wash 4X with TBST. Pat on paper towel to remove excess liquid and bubbles.
9. Add 50 μ L of 1X lysate buffer per well.
- 15 10. Dilute compounds/extracts 1:10 in 1X Kinase Buffer in a polypropylene 96 well plate.
11. Transfer 5.5 μ L of diluted drug to ELISA plate wells. Incubate at room temperature with shaking for 20 min.
12. Add 5.5 μ L of 60 μ M ATP solution per well. Negative controls do not receive any ATP. Incubate at room temperature for 90 min., with shaking.
- 20 13. Wash 4X with TBST. Pat plate on paper towel to remove excess liquid and bubbles.
14. Add 100 μ L per well of RC20 (1:3000 dilution in Blocking Buffer). Incubate 30 min. at room temperature with shaking.
- 25 15. Wash 4X with TBST. Pat plate on paper towel to remove excess liquid and bubbles.
16. Add 100 μ L per well of Turbo-TMB. Incubate with shaking for 30-60 min.
17. Add 100 μ L per well of 1 M H_2SO_4 to stop reaction.
18. Read assay on Dynatech MR7000 ELISA reader. Test Filter = 450 nm,
- 30 reference filter = 410 nm.

EXAMPLE 12: Biochemical src assay - ELISA

This assay is used to determine src protein kinase activity measuring phosphorylation of a biotinylated peptide as the readout.

5 Materials and Reagents

The following materials and reagents were used:

- a. Yeast transformed with.
- b. Cell lysates: Yeast cells expressing src are pelleted, washed once with water, re-pelleted and stored at -80 °C until use.
- 10 c. N-terminus biotinylated EEEYEEYEEEEEEEEEEY is prepared by standard procedures well known to those skilled in the art.
- d. DMSO: Sigma, St. Louis, MO.
- e. 96 Well ELISA Plate: Corning 96 Well Easy Wash, Modified flat Bottom Plate, Corning Cat. #25805-96.
- 15 f. NUNC 96-well V-bottom polypropylene plates for dilution of compounds: Applied Scientific Cat. # A-72092.
- g. Vecastain ELITE ABC reagent: Vector, Burlingame, CA.
- h. Anti-src (327) mab: Schizosaccharomyces Pombe was used to express recombinant Src (Superti-Furga, et al., EMBO J., 12:2625-2634; Superti-Furga, et al., Nature Biochem., 14:600-605). S. Pombe strain SP200 (h-s leul.32 ura4 ade210)
- 20 i. was grown as described and transformations were pRSP expression plasmids were done by the lithium acetate method (Superti-Furga, supra). Cells were grown in the presence of 1 µM thiamine to repress expression from the nmtl promoter or in the absence of thiamine to induce expression.
- 25 i. Monoclonal anti-phosphotyrosine, UBI 05-321 (UB40 may be used instead).
- j. Turbo TMB-ELISA peroxidase substrate: Pierce Chemical.

Buffer Solutions:

- a. PBS (Dulbecco's Phosphate-Buffered Saline): GIBCO PBS, GIBCO Cat. #
- 30 450-1300EB.
- b. Blocking Buffer: 5% Non-fat milk (Carnation) in PBS.

- c. Carbonate Buffer: Na_2CO_3 from Fischer, Cat. # S495, make up 100 mM stock solution.
- d. Kinase Buffer: 1.0 mL (from 1 M stock solution) MgCl_2 ; 0.2 mL (from a 1 M stock solution) MnCl_2 ; 0.2 mL (from a 1 M stock solution) DTT; 5.0 ml (from a 1 M stock solution) HEPES; 0.1 mL TX-100; bring to 10 mL total volume with MilliQ H_2O .
- e. Lysis Buffer: 5.0 HEPES (from 1 M stock solution.); 2.74 mL NaCl (from 5 M stock solution); 10 mL glycerol; 1.0 mL TX-100; 0.4 ml EDTA (from a 100 mM stock solution); 1.0 mL PMSF (from a 100 mM stock solution); 0.1 mL Na_3VO_4 (from a 0.1 M stock solution); bring to 100 mL total volume with MilliQ H_2O .
- f. ATP: Sigma Cat. # A-7699, make up 10 mM stock solution (5.51 mg/mL).
- g. TRIS-HCl: Fischer Cat. # BP 152-5, to 600 ml MilliQ H_2O add 121.14 g material, adjust pH to 7.5 with HCl, bring to 1 L total volume with MilliQ H_2O .
- h. NaCl: Fischer Cat. # S271-10, Make up 5M stock solution with MilliQ H_2O .
- i. Na_3VO_4 : Fischer Cat. # S454-50; to 80 mL MilliQ H_2O , add 1.8 g material; adjust pH to 10.0 with HCl or NaOH; boil in a microwave; cool; check pH, repeat pH adjustment until pH remains stable after heating/cooling cycle; bring to 100 mL total volume with MilliQ H_2O ; make 1 mL aliquots and store at -80°C .
- j. MgCl_2 : Fischer Cat. # M33-500, make up 1M stock solution with MilliQ H_2O .
- k. HEPES: Fischer Cat. # BP 310-500; to 200 mL MilliQ H_2O , add 59.6 g material, adjust pH to 7.5, bring to 250 ml total volume with MilliQ H_2O , sterile filter (1 M stock solution).
- l. TBST Buffer: TBST Buffer: To 900 mL dH_2O add 6.057 g TRIS and 8.766 g NaCl; adjust pH to 7.2 with HCl, add 1.0 mL Triton-X100; bring to 1 L total volume with dH_2O .
- m. MnCl_2 : Fischer Cat. # M87-100, make up 1 M stock solution with MilliQ H_2O .
- n. DTT; Fischer Cat. # BP172-5.
- o. TBS (TRIS Buffered Saline): to 900 mL MilliQ H_2O add 6.057 g TRIS and 8.777 g NaCl; bring to 1 L total volume with MilliQ H_2O .

- p. Kinase Reaction Mixture: Amount per assay plate (100 wells): 1.0 mL Kinase Buffer, 200 µg GST-ζ, bring to final volume of 8.0 mL with MilliQ H₂O.
- q. Biotin labeled EEEYEEYEEYEEYEEY: Make peptide stock solution (1 mM, 2.98 mg/ml) in water fresh just before use.
- 5 r. Vectastain ELITE ABC reagent: To prepare 14 mL of working reagent, add 1 drop of reagent A to 15 mL TBST and invert tube several times to mix. Then add 1 drop of reagent B. Put tube on orbital shaker at room temperature and mix for 30 minutes.
- 10 Procedures
- a. Preparation of src coated ELISA plate.
1. Coat ELISA plate with 0.5 µg/well anti-src mab in 100 µL of pH 9.6 sodium carbonate buffer at 4 °C overnight.
2. Wash wells once with PBS.
- 15 3. Block plate with 0.15 mL 5% milk in PBS for 30 min. at room temperature.
4. Wash plate 5X with PBS.
5. Add 10 µg/well of src transformed yeast lysates diluted in Lysis Buffer (0.1 mL total volume per well). (Amount of lysate may vary between batches.) Shake plate for 20 minutes at room temperature.
- 20 b. Preparation of phosphotyrosine antibody-coated ELISA plate.
1. 4G10 plate: coat 0.5 µg/well 4G10 in 100 µL PBS overnight at 4 °C and block with 150 µL of 5% milk in PBS for 30 minutes at room temperature.
- c. Kinase assay procedure.
1. Remove unbound proteins from step 1-7, above, and wash plates 5X with
- 25 PBS.
2. Add 0.08 mL Kinase Reaction Mixture per well (containing 10 µL of 10X Kinase Buffer and 10 µM (final concentration) biotin-EEYEEYEEYEEYEEY per well diluted in water.
3. Add 10 µL of compound diluted in water containing 10% DMSO and pre-
- 30 incubate for 15 minutes at room temperature.
4. Start kinase reaction by adding 10 µL/well of 0.05 mM ATP in water (5 µM

ATP final).

5. Shake ELISA plate for 15 min. at room temperature.
6. Stop kinase reaction by adding 10 μ L of 0.5 M EDTA per well.
7. Transfer 90 μ L supernatant to a blocked 4G10 coated ELISA plate from section B, above.
8. Incubate for 30 min. while shaking at room temperature.
9. Wash plate 5X with TBST.
10. Incubate with Vectastain ELITE ABC reagent (100 μ L/well) for 30 min. at room temperature.
- 10 11. Wash the wells 5X with TBST.
12. Develop with Turbo TMB.

EXAMPLE 13: Biochemical lck Assay - ELISA

- This assay is used to determine lck protein kinase activities measuring phosphorylation of GST- ζ as the readout.
- 15

Materials and Reagents

The following materials and reagents were used:

- a. Yeast transformed with lck. Schizosaccharomyces Pombe was used to express recombinant Lck (Superti-Furga, et al., EMBO J, 12:2625-2634; Superti-Furga, et al., Nature Biotech., 14:600-605). S. Pombe strain SP200 (h-s leu1.32 ura4 ade210) was grown as described and transformations with pRSP expression plasmids were done by the lithium acetate method (Superti-Furga, supra). Cells were grown in the presence of 1 μ M thiamine to induce expression.
- 20 b. Cell lysates: Yeast cells expressing lck are pelleted, washed once in water, re-pelleted and stored frozen at -80 °C until use.
- c. GST- ζ : DNA encoding for GST- ζ fusion protein for expression in bacteria obtained from Arthur Weiss of the Howard Hughes Medical Institute at the University of California, San Francisco. Transformed bacteria were grown overnight while shaking at 25 °C. GST- ζ was purified by glutathione affinity chromatography, Pharmacia, Alameda, CA.
- 25
- 30

- d. DMSO: Sigma, St. Louis, MO.
- e. 96-Well ELISA plate: Corning 96 Well Easy Wash, Modified Flat Bottom Plate, Corning Cat. #25805-96.
- f. NUNC 96-well V-bottom polypropylene plates for dilution of compounds:
5 Applied Scientific Cat. # AS-72092.
- g. Purified Rabbit anti-GST antiserum: Amrad Corporation (Australia) Cat. #90001605.
- h. Goat anti-Rabbit-IgG-HRP: Amersham Cat. # V010301
- i. Sheep anti-mouse IgG (H+L): Jackson Labs Cat. # 5215-005-003.
- 10 j. Anti-Lck (3A5) mab: Santa Cruz Biotechnology Cat # sc-433.
- k. Monoclonal anti-phosphotyrosine UBI 05-321 (UB40 may be used instead).

Buffer solutions:

- a. PBS (Dulbecco's Phosphate-Buffered Saline) 1X solution: GIBCO PBS,
15 GIBCO Cat. # 450-1300EB.
- b. Blocking Buffer: 100 g. BSA, 12.1 g. TRIS-pH7.5, 58.44 g NaCl, 10 mL Tween-20, bring up to 1 L total volume with MilliQ H₂O.
- c. Carbonate Buffer: Na₂CO₃ from Fischer, Cat. # S495; make up 100 mM solution with MilliQ H₂O.
- 20 d. Kinase Buffer: 1.0 mL (from 1 M stock solution) MgCl₂; 0.2 mL (from a 1 M stock solution) MnCl₂; 0.2 mL (from a 1 M stock solution) DTT; 5.0 mL (from a 1 M stock solution) HEPES; 0.1 mL TX-100; bring to 10 mL total volume with MilliQ H₂O.
- e. Lysis Buffer: 5.0 HEPES (from 1 M stock solution.); 2.74 mL NaCl (from 5
25 M stock solution); 10 mL glycerol; 1.0 mL TX-100; 0.4 mL EDTA (from a 100 mM stock solution); 1.0 mL PMSF (from a 100 mM stock solution); 0.1 mL Na₃VO₄ (from a 0.1 M stock solution); bring to 100 mL total volume with MilliQ H₂O.
- f. ATP: Sigma Cat. # A-7699, make up 10 mM stock solution (5.51 mg/mL).
- g. TRIS-HCl: Fischer Cat. # BP 152-5, to 600 mL MilliQ H₂O add 121.14 g
30 material, adjust pH to 7.5 with HCl, bring to 1 L total volume with MilliQ H₂O.
- h. NaCl: Fischer Cat. # S271-10, Make up 5 M stock solution with MilliQ

H₂O.

- i. Na₃VO₄: Fischer Cat. # S454-50; to 80 mL MilliQ H₂O, add 1.8 g material; adjust pH to 10.0 with HCl or NaOH; boil in a microwave; cool; check pH, repeat pH adjustment until pH remains stable after heating/cooling cycle; bring to 100 ml total
5 volume with MilliQ H₂O; make 1 ml aliquots and store at -80 °C.
- j. MgCl₂: Fischer Cat. # M33-500, make up 1M stock solution with MilliQ H₂O.
- k. HEPES: Fischer Cat. # BP 310-500; to 200 mL MilliQ H₂O, add 59.6 g material, adjust pH to 7.5, bring to 250 mL total volume with MilliQ H₂O, sterile
10 filter (1M stock solution).
- l. Albumin, Bovine (BSA), Sigma Cat. # A4503; to 150 mL MilliQ H₂O add 30 g material, bring 300 mL total volume with MilliQ H₂O, filter through 0.22 µm filter, store at 4 °C.
- m. TBST Buffer: To 900 mL dH₂O add 6.057 g TRIS and 8.766 g NaCl; adjust
15 pH to 7.2 with HCl, add 1.0 mL Triton-X100; bring to 1 L total volume with dH₂O.
- n. MnCl₂: Fischer Cat. # M87-100, make up 1 M stock solution with MilliQ H₂O.
- o. DTT; Fischer Cat. # BP172-5.
- p. TBS (TRIS Buffered Saline): to 900 mL MilliQ H₂O add 6.057 g TRIS and
20 8.777 g NaCl; bring to 1 L total volume with MilliQ H₂O.
- q Kinase Reaction Mixture: Amount per assay plate (100 wells): 1.0 mL Kinase Buffer, 200 µg GST-ζ, bring to final volume of 8.0 mL with MilliQ H₂O.

Procedures

- 25 a. Preparation of Lck coated ELISA plate.
 1. Coat 2.0 µg/well Sheep anti-mouse-IgG in 100 µL of pH 9.6 sodium carbonate buffer at 4 °C overnight.
 2. Wash well once with PBS.
 3. Block plate with 0.15 mL of blocking Buffer for 30 min. at room temp.
 - 30 4. Wash plate 5X with PBS.
 5. Add 0.5 µg/well of anti-lck (mab 3A5) in 0.1 mL PBS at room temperature

for 1-2 hours.

6. Wash plate 5X with PBS.

7. Add 20 µg/well of lck transformed yeast lysates diluted in Lysis Buffer (0.1 mL total volume per well). (Amount of lysate may vary between batches) Shake

5 plate at 4 °C overnight to prevent loss of activity.

b. Preparation of phosphotyrosine antibody-coated ELISA plate.

1. UB40 plate: 1.0 µg/well UB40 in 100 µL of PBS overnight at 4 °C and block with 150 µL of Blocking Buffer for at least 1 hour.

10 c. Kinase assay procedure.

1. Remove unbound proteins from step 1-7, above, and wash plates 5X with PBS.

2. Add 0.08 mL Kinase Reaction Mixture per well (containing 10 µL of 10X Kinase Buffer and 2 µg GST-ζ per well diluted with water).

15 3. Add 10 µL of compound diluted in water containing 10% DMSO and pre-incubate for 15 minutes at room temperature.

4. Start kinase reaction by adding 10 µL/well of 0.1 mM ATP in water (10 µM ATP final).

5. Shake ELISA plate for 60 min. at room temperature.

20 6. Stop kinase reaction by adding 10 µL of 0.5 M EDTA per well.

7. Transfer 90 µL supernatant to a blocked 4G10 coated ELISA plate from section B, above.

8. Incubate while shaking for 30 min. at room temperature.

9. Wash plate 5X with TBST.

25 10. Incubate with Rabbit anti-GST antibody at 1:5000 dilution in 100 µL TBST for 30 min. at room temperature.

11. Wash the wells 5X with TBST.

12. Incubate with Goat anti-Rabbit-IgG-HRP at 1:20,000 dilution in 100 µL of TBST for 30 min. at room temperature.

30 13. Wash the wells 5X with TBST.

14. Develop with Turbo TMB.

EXAMPLE 14: ASSAY MEASURING PHOSPHORYLATING FUNCTION
OF RAF

5 The following assay reports the amount of RAF-catalyzed phosphorylation of its target protein MEK as well as MEK's target MAPK. The RAF gene sequence is described in Bonner et al., 1985, Molec. Cell. Biol. 5: 1400-1407, and is readily accessible in multiple gene sequence data banks. Construction of the nucleic acid vector and cell lines utilized for this portion of the invention are fully described in Morrison et al., 1988, Proc. Natl. Acad. Sci. USA 85: 8855-8859.

10

Materials and Reagents

1. *Sf9* (*Spodoptera frugiperda*) cells; GIBCO-BRL, Gaithersburg, MD.
2. RIPA buffer: 20 mM Tris/HCl pH 7.4, 137 mM NaCl, 10% glycerol, 1 mM PMSF, 5 mg/L Aprotinin, 0.5 % Triton X-100;
- 15 3. Thioredoxin-MEK fusion protein (T-MEK): T-MEK expression and purification by affinity chromatography were performed according to the manufacturer's procedures. Catalog# K 350-01 and R 350-40, Invitrogen Corp., San Diego, CA
4. His-MAPK (ERK 2); His-tagged MAPK was expressed in XL1 Blue cells
20 transformed with pUC18 vector encoding His-MAPK. His-MAPK was purified by Ni-affinity chromatography. Cat# 27-4949-01, Pharmacia, Alameda, CA, as described herein.
5. Sheep anti mouse IgG: Jackson laboratories, West Grove, PA. Catalog, # 515-006-008, Lot# 28563
- 25 6. RAF-1 protein kinase specific antibody: URP2653 from UBI.
7. Coating buffer: PBS; phosphate buffered saline, GIBCO-BRL, Gaithersburg, MD
8. Wash buffer: TBST - 50 mM Tris/HCL pH 7.2, 150 mM NaCl, 0.1 % Triton X-100
- 30 9. Block buffer: TBST, 0.1 % ethanolamine pH 7.4
10. DMSO, Sigma, St. Louis, MO

11. Kinase buffer (KB): 20 mM HEPES/HCl pH 7.2, 150 mM NaCl, 0.1 % Triton X-100, 1 mM PMSF, 5 mg/L Aprotinin, 75 mM sodium ortho vanadate, 0.5 mM DTT and 10 mM MgCl₂.
12. ATP mix: 100 mM MgCl₂, 300 mM ATP, 10 mCi ³³P ATP (Dupont-
5 NEN)/mL.
13. Stop solution: 1 % phosphoric acid; Fisher, Pittsburgh, PA.
14. Wallac Cellulose Phosphate Filter mats; Wallac, Turku, Finland.
15. Filter wash solution: 1 % phosphoric acid, Fisher, Pittsburgh, PA.
16. Tomtec plate harvester, Wallac, Turku, Finland.
- 10 17. Wallac beta plate reader # 1205, Wallac, Turku, Finland.
18. NUNC 96-well V bottom polypropylene plates for compounds Applied Scientific Catalog # AS-72092.

Procedure

- 15 All of the following steps were conducted at room temperature unless specifically indicated.
1. ELISA plate coating: ELISA wells are coated with 100 mL of Sheep anti mouse affinity purified antiserum (1 mg/100 mL coating buffer) over night at 4 °C. ELISA plates can be used for two weeks when stored at 4 °C.
- 20 2. Invert the plate and remove liquid. Add 100 mL of blocking solution and incubate for 30 min.
3. Remove blocking solution and wash four times with wash buffer. Pat the plate on a paper towel to remove excess liquid.
4. Add 1 mg of antibody specific for RAF-1 to each well and incubate for 1
25 hour. Wash as described in step 3.
5. Thaw lysates from RAS/RAF infected Sf9 cells and dilute with TBST to 10 mg/100 mL. Add 10 mg of diluted lysate to the wells and incubate for 1 hour. Shake the plate during incubation. Negative controls receive no lysate. Lysates from RAS/RAF infected Sf9 insect cells are prepared after cells are infected with
30 recombinant baculoviruses at a MOI of 5 for each virus, and harvested 48 hours later. The cells are washed once with PBS and lysed in RIPA buffer. Insoluble

material is removed by centrifugation (5 min at 10 000 x g). Aliquots of lysates are frozen in dry ice/ethanol and stored at -80 °C until use.

6. Remove non-bound material and wash as outlined above (step 3).
7. Add 2 mg of T-MEK and 2 mg of His-MAEPK per well and adjust the
5 volume to 40 mL with kinase buffer. Methods for purifying T-MEK and MAPK from cell extracts are provided herein by example.
8. Pre-dilute compounds (stock solution 10 mg/mL DMSO) or extracts 20 fold in TBST plus 1% DMSO. Add 5 mL of the pre-diluted compounds/extracts to the wells described in step 6. Incubate for 20 min. Controls receive no drug.
- 10 9. Start the kinase reaction by addition of 5 mL ATPmix; Shake the plates on an ELISA plate shaker during incubation.
10. Stop the kinase reaction after 60 min by addition of 30 mL stop solution to each well.
11. Place the phosphocellulose mat and the ELISA plate in the Tomtec plate
15 harvester. Harvest and wash the filter with the filter wash solution according to the manufacturers recommendation. Dry the filter mats. Seal the filter mats and place them in the holder. Insert the holder into radioactive detection apparatus and quantify the radioactive phosphorous on the filter mats.

- Alternatively, 40 mL aliquots from individual wells of the assay plate can be
20 transferred to the corresponding positions on the phosphocellulose filter mat. After air drying the filters, put the filters in a tray. Gently rock the tray, changing the wash solution at 15 min intervals for 1 hour. Air-dry the filter mats. Seal the filter mats and place them in a holder suitable for measuring the radioactive phosphorous in the samples. Insert the holder into a detection device and quantify the radioactive
25 phosphorous on the filter mats.

Cellular/Biologic Assays

EXAMPLE 15: GENERAL PROCEDURE FOR BRDU INCORPORATION 30 ASSAYS

The following assays use cells engineered to express a receptor of interest

and the evaluate the effect of a compound of interest on the activity of ligand-induced DNA synthesis by determining BrdU incorporation into the DNA.

The following materials, reagents, and procedure are general to each of the following BrdU incorporation assays. Variances in specific assays are noted.

5

Materials and Reagents:

1. The appropriate ligand.
2. The appropriate engineered cells.
3. BrdU Labeling Reagent: 10 mM, in PBS (pH7.4) (Boehringer Mannheim,
10 Germany).
4. FixDenat: fixation solution (ready to use) (Boehringer Mannheim, Germany).
5. Anti-BrdU-POD: mouse monoclonal antibody conjugated with peroxidase (Boehringer Mannheim, Germany).
6. TMB Substrate Solution: tetramethylbenzidine (TMB, Boehringer
15 Mannheim, Germany).
7. PBS Washing Solution : 1X PBS, pH 7.4.
8. Albumin, Bovine (BSA), fraction V powder (Sigma Chemical Co., USA).

General Procedure:

- 20 1. Cells are seeded at 8000 cells/well in 10% CS, 2mM Gln in DMEM, in a 96 well plate. Cells are incubated overnight at 37 °C in 5% CO₂.
2. After 24 hours, the cells are washed with PBS, and then are serum starved in serum free medium (0%CS DMEM with 0.1% BSA) for 24 hours.
3. On day 3, the appropriate ligand and the test compound are added to the cells
25 simultaneously. The negative control wells receive serum free DMEM with 0.1% BSA only; the positive control cells receive the ligand but no test compound. Test compounds are prepared in serum free DMEM with ligand in a 96 well plate, and serially diluted for 7 test concentrations.
4. After 18 hours of ligand activation, diluted BrdU labeling reagent (1:100 in
30 DMEM, 0.1% BSA) is added and the cells are incubated with BrdU (final concentration=10 µM) for 1.5 hours.

5. After incubation with labeling reagent, the medium is removed by decanting and tapping the inverted plate on a paper towel. FixDenat solution is added (50 $\mu\text{L}/\text{well}$) and the plates are incubated at room temperature for 45 minutes on a plate shaker.
- 5 6. The FixDenat solution is thoroughly removed by decanting and tapping the inverted plate on a paper towel. Milk is added (5% dehydrated milk in PBS, 200 $\mu\text{L}/\text{well}$) as a blocking solution and the plate is incubated for 30 minutes at room temperature on a plate shaker.
7. The blocking solution is removed by decanting and the wells are washed
10 once with PBS. Anti-BrdU-POD solution (1:200 dilution in PBS, 1% BSA) is added (50 $\mu\text{L}/\text{well}$) and the plate is incubated for 90 minutes at room temperature on a plate shaker.
8. The antibody conjugate is thoroughly removed by decanting and rinsing the wells 5 times with PBS, and the plate is dried by inverting and tapping on a paper
15 towel.
9. TMB substrate solution is added (100 $\mu\text{L}/\text{well}$) and incubated for 20 minutes at room temperature on a plate shaker until color development is sufficient for photometric detection.
10. The absorbance of the samples are measured at 410 nm (in "dual
20 wavelength" mode with a filter reading at 490 nm, as a reference wavelength) on a Dynatech ELISA plate reader.

EXAMPLE 16: EGF-Induced BrdU Incorporation Assay

- The procedure is the same as the general procedure, outlined above, except
25 for the following changes:

Materials and Reagents:

1. Mouse EGF, 201 (Toyobo Co., Ltd., Japan).
 2. 3T3/EGFRc7.
- 30

EXAMPLE 17: EGF-Induced Her-2-driven BrdU Incorporation Assay

The procedure is the same as the general procedure, outlined above, except for the following changes:

5 Materials and Reagents:

1. Mouse EGF, 201 (Toyobo Co., Ltd., Japan).
2. 3T3/EGFr/Her2/EGFr (EGFr with a Her-2 kinase domain).

EXAMPLE 18: EGF-Induced Her-4-driven BrdU Incorporation Assay

10 The procedure is the same as the general procedure, outlined above, except for the following changes:

Materials and Reagents:

1. Mouse EGF, 201 (Toyobo Co., Ltd., Japan).
- 15 2. 3T3/EGFr/Her4/EGFr (EGFr with a Her-4 kinase domain).

EXAMPLE 19: PDGF-Induced BrdU Incorporation Assay

The procedure is the same as the general procedure, outlined above, except for the following changes:

20

Materials and Reagents:

1. Human PDGF B/B (Boehringer Mannheim, Germany).
2. 3T3/EGFRc7.

25 EXAMPLE 20: FGF-Induced BrdU Incorporation Assay

The procedure is the same as the general procedure, outlined above, except for the following changes:

Materials and Reagents:

1. Human FGF2/bFGF (Gibco BRL, USA).
2. 3T3c7/EGFr

5 EXAMPLE 21: IGF1-Induced BrdU Incorporation Assay

The procedure is the same as the general procedure, outlined above, except for the following changes:

Materials and Reagents:

- 10
1. Human, recombinant (G511, Promega Corp., USA)
 2. 3T3/IGF1r.

EXAMPLE 22: Insulin-Induced BrdU Incorporation Assay

- 15
- The procedure is the same as the general procedure, outlined above, except for the following changes:

Materials and Reagents:

1. Insulin, crystalline, bovine, Zinc (13007, Gibco BRL, USA).

20 EXAMPLE 23: HGF-Induced BrdU Incorporation Assay

The procedure is the same as the general procedure, outlined above, except for the following changes:

Materials and Reagents:

- 25
1. Recombinant human HGF (Cat. No. 249-HG, R&D Systems, Inc. USA).
 2. BxPC-3 cells (ATCC CRL-1687).

Procedure:

1. Cells are seeded at 9000 cells/well in RPMI 10% FBS in a 96 well plate.

Cells are incubated overnight at 37°C in 5% CO₂.

2. After 24 hours, the cells are washed with PBS, and then are serum starved in 100 µl serum-free medium (RPMI with 0.1% BSA) for 24 hours.
3. On day 3, 25 µl containing ligand (prepared at 1 µg/ml in RPMI with 0.1% BSA; final HGF conc. = 200 ng/ml) and test compounds are added to the cells. The negative control wells receive 25 µl serum-free RPMI with 0.1% BSA only; the positive control cells receive the ligand (HGF) but no test compound. Test compounds are prepared at 5 times their final concentration in serum-free RPMI with ligand in a 96 well plate, and serially diluted for 7 test concentrations.
- 10 Typically, the highest final concentration of test compound is 100 µM, and 1:3 dilutions are used (i.e. final test compound concentration range = 0.137-100 µM).
4. After 18 hours of ligand activation, 12.5 µl of diluted BrdU labeling reagent (1:100 in RPMI, 0.1% BSA) is added to each well and the cells are incubated with BrdU (final concentration = 10 µM) for 1 hour.
- 15 5. Same as General Procedure.
6. Same as General Procedure.
7. The blocking solution is removed by decanting and the wells are washed once with PBS. Anti-BrdU-POD solution (1:100 dilution in PBS, 1% BSA) is added (100 µl/well) and the plate is incubated for 90 minutes at room temperature on a plate shaker.
- 20 8. Same as General Procedure.
9. Same as General Procedure.
10. Same as General Procedure.

25 EXAMPLE 24: HUV-EC-C Assay

The following protocol may also be used to measure a compound's activity against PDGF-R, FGF-R, VEGF, aFGF or Flk-1/KDR, all of which are naturally expressed by HUV-EC cells.

30 DAY 0

1. Wash and trypsinize HUV-EC-C cells (human umbilical vein

endothelial cells, (American Type Culture Collection; catalogue no. 1730 CRL).

Wash with Dulbecco's phosphate-buffered saline (D-PBS; obtained from Gibco BRL; catalogue no. 14190-029) 2 times at about 1 mL/10 cm² of tissue culture flask.

- Trypsinize with 0.05% trypsin-EDTA in non-enzymatic cell dissociation solution (Sigma Chemical Company; catalogue no. C-1544). The 0.05% trypsin was made by diluting 0.25% trypsin/1 mM EDTA (Gibco; catalogue no. 25200-049) in the cell dissociation solution. Trypsinize with about 1 mL/25-30 cm² of tissue culture flask for about 5 minutes at 37 °C. After cells have detached from the flask, add an equal volume of assay medium and transfer to a 50 mL sterile centrifuge tube (Fisher Scientific; catalogue no. 05-539-6).

2. Wash the cells with about 35 mL assay medium in the 50 mL sterile centrifuge tube by adding the assay medium, centrifuge for 10 minutes at approximately 200 g, aspirate the supernatant, and resuspend with 35 mL D-PBS. Repeat the wash two more times with D-PBS, resuspend the cells in about 1 mL assay medium/15 cm² of tissue culture flask. Assay medium consists of F12K medium (Gibco BRL; catalogue no. 21127-014) + 0.5% heat-inactivated fetal bovine serum. Count the cells with a Coulter Counter (Coulter Electronics, Inc.) and add assay medium to the cells to obtain a concentration of $0.8-1.0 \times 10^5$ cells/mL.

3. Add cells to 96-well flat-bottom plates at 100 µL/well or $0.8-1.0 \times 10^4$ cells/well; incubate ~24h at 37 °C, 5% CO₂.

DAY 1

1. Make up two-fold drug titrations in separate 96-well plates, generally 50 µM on down to 0 µM. Use the same assay medium as mentioned in day 0, step 2 above. Titrations are made by adding 90 µL/well of drug at 200 µM (4X the final well concentration) to the top well of a particular plate column. Since the stock drug concentration is usually 20 mM in DMSO, the 200 µM drug concentration contains 2% DMSO.

Therefore, diluent made up to 2% DMSO in assay medium (F12K + 0.5% fetal bovine serum) is used as diluent for the drug titrations in order to dilute the drug but keep the DMSO concentration constant. Add this diluent to the remaining

- wells in the column at 60 μ L/well. Take 60 μ L from the 120 μ L of 200 μ M drug dilution in the top well of the column and mix with the 60 μ L in the second well of the column. Take 60 μ L from this well and mix with the 60 μ L in the third well of the column, and so on until two-fold titrations are completed. When the next-to-the
- 5 last well is mixed, take 60 μ L of the 120 μ L in this well and discard it. Leave the last well with 60 μ L of DMSO/media diluent as a non-drug-containing control. Make 9 columns of titrated drug, enough for triplicate wells each for 1) VEGF (obtained from Pepro Tech Inc., catalogue no. 100-200, 2) endothelial cell growth factor (ECGF) (also known as acidic fibroblast growth factor, or aFGF) (obtained
- 10 from Boehringer Mannheim Biochemica, catalogue no. 1439 600); or, 3) human PDGF B/B (1276-956, Boehringer Mannheim, Germany) and assay media control. ECGF comes as a preparation with sodium heparin.
2. Transfer 50 μ L/well of the drug dilutions to the 96-well assay plates containing the $0.8-1.0 \times 10^4$ cells/100 μ L/well of the HUV-EC-C cells from day 0 and
- 15 incubate ~ 2 h at 37 $^{\circ}$ C, 5% CO₂.
3. In triplicate, add 50 μ L/well of 80 μ g/mL VEGF, 20 ng/mL ECGF, or media control to each drug condition. As with the drugs, the growth factor concentrations are 4X the desired final concentration. Use the assay media from day 0 step 2 to make the concentrations of growth factors. Incubate approximately 24
- 20 hours at 37 $^{\circ}$ C, 5% CO₂. Each well will have 50 μ L drug dilution, 50 μ L growth factor or media, and 100 μ L cells, = 200 μ L/well total. Thus the 4X concentrations of drugs and growth factors become 1X once everything has been added to the wells.

DAY 2

- 25 1. Add ³H-thymidine (Amersham; catalogue no. TRK-686) at 1 μ Ci/well (10 μ L/well of 100 μ Ci/mL solution made up in RPMI media + 10% heat-inactivated fetal bovine serum) and incubate ~ 24 h at 37 $^{\circ}$ C, 5% CO₂. Note: ³H-thymidine is made up in RPMI media because all of the other applications for which we use the ³H-thymidine involve experiments done in RPMI. The media difference
- 30 at this step is probably not significant. RPMI was obtained from Gibco BRL, catalogue no. 11875-051.

DAY 3

1. Freeze plates overnight at -20 °C.

DAY 4

1. Thaw plates and harvest with a 96-well plate harvester (Tomtec Harvester 96^(R)) onto filter mats (Wallac; catalogue no. 1205-401); read counts on a Wallac Betaplate^(TM) liquid scintillation counter.

In Vivo Animal Models10 EXAMPLE 25: Xenograft Animal Models

The ability of human tumors to grow as xenografts in athymic mice (*e.g.*, Balb/c, nu/nu) provides a useful *in vivo* model for studying the biological response to therapies for human tumors. Since the first successful xenotransplantation of human tumors into athymic mice, (Rygaard and Povlsen, 1969, Acta Pathol.

- 15 Microbial. Scand. 77:758-760), many different human tumor cell lines (*e.g.*, mammary, lung, genitourinary, gastro-intestinal, head and neck, glioblastoma, bone, and malignant melanomas) have been transplanted and successfully grown in nude mice. The following assays may be used to determine the level of activity, specificity and effect of the different compounds of the present invention.

- 20 Suitable cell lines for subcutaneous xenograft experiments include C6 cells (glioma, ATCC # CCL 107), A375 cells (melanoma, ATCC # CRL 1619), A431 cells (epidermoid carcinoma, ATCC # CRL 1555), Calu 6 cells (lung, ATCC # HTB 56), PC3 cells (prostate, ATCC # CRL 1435) and NIH 3T3 fibroblasts genetically engineered to overexpress EGFR, PDGFR, IGF-1R or any other test kinase. The
- 25 following protocol can be used to perform xenograft experiments:

Female athymic mice (BALB/c, nu/nu) are obtained from Simonsen Laboratories (Gilroy, CA). All animals are maintained under clean-room conditions in Micro-isolator cages with Alpha-dri bedding. They receive sterile rodent chow and water *ad libitum*.

- 30 Cell lines are grown in appropriate medium (for example, MEM, DMEM, Ham's F10, or Ham's F12 plus 5% - 10% fetal bovine serum (FBS) and 2 mM

glutamine (GLN)). All cell culture media, glutamine, and fetal bovine serum are purchased from Gibco Life Technologies (Grand Island, NY) unless otherwise specified. All cells are grown in a humid atmosphere of 90-95% air and 5-10% CO₂ at 37 °C. All cell lines are routinely subcultured twice a week and are
5 negative for mycoplasma as determined by the Mycotect method (Gibco).

Cells are harvested at or near confluency with 0.05% Trypsin-EDTA and pelleted at 450 x g for 10 min. Pellets are resuspended in sterile PBS or media (without FBS) to a particular concentration and the cells are implanted into the hindflank of the mice (8 - 10 mice per group, 2 - 10 x 10⁶ cells/animal). Tumor
10 growth is measured over 3 to 6 weeks using venier calipers. Tumor volumes are calculated as a product of length x width x height unless otherwise indicated. P values are calculated using the Students t-test. Test compounds in 50 - 100 µL excipient (*e.g.*, DMSO, or VPD:D5W) are delivered by IP injection at different concentrations generally starting at day one after implantation.

15

EXAMPLE 26: Tumor Invasion Model

The following tumor invasion model has been developed and maybe used for the evaluation of therapeutic value and efficacy of the compounds identified to selectively inhibit KDR/FLK-1 receptor or CDK2.

20

Procedure

8 week old nude mice (female) (Simonsen Inc.) were used as experimental animals. Implantation of tumor cells was performed in a laminar flow hood. For anesthesia, Xylazine/Ketamine Cocktail (100 mg/kg ketamine and 5 mg/kg
25 Xylazine) are administered intraperitoneally. A midline incision is done to expose the abdominal cavity (approximately 1.5 cm in length) to inject 10⁷ tumor cells in a volume of 100 µL medium. The cells are injected either into the duodenal lobe of the pancreas or under the serosa of the colon. The peritoneum and muscles are closed with a 6-0 silk continuous suture and the skin was closed
30 by using wound clips. Animals were observed daily.

Analysis

After 2-6 weeks, depending on gross observations of the animals, the mice are sacrificed, and the local tumor metastases, to various organs (lung, liver, brain, stomach, spleen, heart, muscle) are excised and analyzed (measurements of tumor size, grade of invasion, immunochemistry, and in situ hybridization).

EXAMPLE 27: Measurement of Cell Toxicity

Therapeutic compounds should be more potent in inhibiting protein kinase activity than in exerting a cytotoxic effect. A measure of the effectiveness and cell toxicity of a compound can be obtained by determining the therapeutic index: IC_{50}/LD_{50} . IC_{50} , the dose required to achieve 50% inhibition, can be measured using standard techniques such as those described herein. LD_{50} , the dosage which results in 50% toxicity, can also be measured by standard techniques (Mossman, 1983, J. Immunol. Methods, 65:55-63), by measuring the amount of LDH released (Korzeniewski and Callewaert, 1983, J. Immunol. Methods, 64:313; Decker and Lohmann-Matthes, 1988, J. Immunol. Methods, 115:61), or by measuring the lethal dose in animal models. Compounds with a large therapeutic index are preferred. The therapeutic index should be greater than 2, preferably at least 10, more preferably at least 50.

EXAMPLE 28: The Activity of the Compounds of the Invention

The biological or biochemical activity of some of the compounds of the invention were tested using the assays described above. The IC_{50} values were measured for several of the compounds of the invention. The results are shown in Table 4 below.

Table 4

Compound #	bio FLK-GST IC_{50} (mM)	bio EGF IC_{50} (mM)	bio PDGF IC_{50} (mM)	CDK2-SPA IC_{50} (mM)
IN-001		>20		0.0055
IN-002	6.64	>20	>20	0.005

Compound #	bio FLK-GST IC ₅₀ (mM)	bio EGF IC ₅₀ (mM)	bio PDGF IC ₅₀ (mM)	CDK2-SPA IC ₅₀ (mM)
IN-003	>20	>20	>20	0.27
IN-004	>20	>20	>20	0.04
IN-005	>20		1.31	1.55
IN-006	15.47	>20	17.55	0.24
IN-007	>20	>20	2.08	0.09
IN-008	2.79	>20	>20	0.03
IN-009	3.91	>20	>20	3.84
IN-010	6.59	>20	>20	0.01
IN-011	>20	>20	>20	0.03
IN-012	16.54	>20	>20	0.06
IN-013	4.24	>20	>20	0.04
IN-014	9.66	>20	10.58	7.86
IN-015	2.58	>20	1.18	>10
IN-016	11.7	>20	>20	0.08
IN-017	11.13	>20	>20	0.004
IN-018	10.13	>20	7.44	1.08
IN-019	>20	>20	>20	0.41
IN-020	>20	>20	5.90	0.97
IN-021	5.77	>20	>20	>10
IN-022	4.30	>20	10.88	>10
IN-023	1.83	>20	>20	1.00
IN-024	5.26	>20	>20	1.06
IN-025	>10	>20	2.88	5.69
IN-026	10.95	>20	>20	0.35
IN-027	5.54	>20	>20	0.55
IN-028	16.64	>20	>20	0.73
IN-029	3.42	>20	>20	2.86
IN-030	4.86	>20	>20	9.95
IN-031	19.24	>20	>20	0.36
IN-032	>20	>20	>20	0.31
IN-033	>20	>20	>20	2.58
IN-034	1.13	>20	>20	6.71
IN-035	11.01	18.67	>20	5.36
IN-036	4.63	>20	>20	0.49
IN-037	4.51	>20	>20	0.16

Compound #	bio FLK-GST IC ₅₀ (mM)	bio EGF IC ₅₀ (mM)	bio PDGF IC ₅₀ (mM)	CDK2-SPA IC ₅₀ (mM)
IN-038		>20	>20	3.20
IN-039		>20	0.04	6.01
IN-040		>20	0.06	>10
IN-041		>20	0.12	7.23
IN-042		>20	19.64	0.10
IN-043		>20	14.39	0.09
IN-044		>20	8.97	0.07
IN-045		>20	18.83	0.56
IN-046		>20	2.02	0.45
IN-047		>20	5.32	0.29
IN-048		>20	<0.009	0.30
IN-049		>20	0.21	0.53
IN-050	1.60			
IN-051		>20	12.08	<0.005
IN-052		>20	6.00	0.009
IN-053		>20	>20	0.13
IN-054	0.19			
IN-055	2.62			
IN-056	1.26			
IN-057	14.29			
IN-058	17.09			

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The molecular complexes and the methods, procedures, treatments, molecules, specific compounds described herein are presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated
5 by reference.

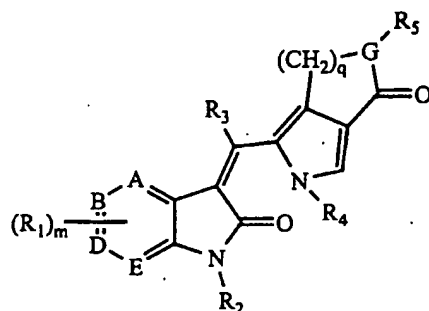
The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising," "consisting essentially of" and "consisting of" may be replaced
10 with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus,
15 it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

In addition, where features or aspects of the invention are described in terms
20 of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. For example, if X is described as selected from the group consisting of bromine, chlorine, and iodine, claims for X being bromine and claims
25 for X being bromine and chlorine are fully described.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the
30 genus, regardless of whether or not the excised material is specifically recited herein.

What is claimed is:

1. A compound of Formula (I):



(I)

wherein:

- (a) each R₁ is independently and optionally selected from the group consisting of:
- (i) hydrogen;
 - 10 (ii) saturated or unsaturated alkyl optionally substituted with one, two or three substituents selected from the group consisting of halogen, trihalomethyl, alkoxy, carboxylate, amino, nitro, ester, and a five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where each ring is optionally substituted with one, two, or three substituents independently selected from the
 - 15 group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;
 - (iii) an aromatic or heteroaromatic ring optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;
 - 20 (iv) an aliphatic or heteroaliphatic ring optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, ester, and an aromatic or heteroaromatic ring optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen,
 - 25 trihalomethyl, carboxylate, amino, nitro, and ester;

- (v) an alcohol of formula $-(X_1)_{n1}-OH$ or an alkoxyalkyl of formula $-(X_2)_{n2}-O-X_3$, where
- X_1 , X_2 , and X_3 are independently selected from the group consisting of saturated or unsaturated alkyl, amide, and five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where the alkyl, and each ring is optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, alkoxy, aryl, heteroaryl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester; and $n1$ and $n2$ are independently 0 or 1;
- (vi) a halogen or trihalomethyl;
- (vii) a carboxylic acid of formula $-(X_4)_{n4}-COOH$ or ester of formula $-(X_5)_{n5}-COO-X_6$, where X_4 , X_5 , and X_6 are independently selected from the group consisting of alkyl and five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring; and $n4$ and $n5$ are independently 0 or 1;
- (viii) an amide or thioamide of formula $-(X_7)_{n7}-NHCOX_8$, $-(X_7)_{n7}-NHCSX_8$, $-(X_9)_{n9}-CONX_{10}X_{11}$, or of formula $-(X_9)_{n9}-CSNX_{10}X_{11}$, where X_7 and X_9 are each independently selected from the group consisting of alkyl and five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where each of the ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester; $n7$ and $n9$ are independently 0 or 1; and X_8 , X_{10} , and X_{11} are each independently selected from the group consisting of hydrogen, alkyl, hydroxyl, alkoxy, and five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where the ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, thioalkyl, aryl, heteroaryl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester; or where X_{10} and X_{11} taken together form a five-membered or six-membered aliphatic or heteroaliphatic ring or a fused bicyclic ring optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;
- (ix) a sulfonamide of formula $-(X_{12})_{n12}-SO_2NX_{13}X_{14}$ or of formula $-(X_{12})_{n12}-NX_{13}-SO_2-X_{14}$, where X_{12} is selected from the group consisting of alkyl, and

- five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where the alkyl and each ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, halogen, hydroxy, trihalomethyl, carboxylate, amino, nitro, and ester; n_{12} is 0, 1, or 2; and X_{13} , and X_{14} are independently selected from the group consisting of hydrogen, alkyl, and three-, four-, five-, or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where the alkyl and each ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, aryl, heteroaryl, halogen, trihalomethyl, aldehyde, carboxylate, amino, nitro, and ester; or where X_{13} and X_{14} taken together form a five-membered or six-membered aliphatic or heteroaliphatic ring or a fused bicyclic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;
- 15 (x) a sulfone of formula $-(X_{15})_{n_{15}}-SO_3H$, where X_{15} is selected from the group consisting of alkyl, and five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where the alkyl and each ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester; n_{15} is 0, 1, or 2; and
- 20 (xi) an amine of formula $-(X_{16})_{n_{16}}-NX_{17}X_{18}$, where X_{16} is selected from the group consisting of saturated or unsaturated alkyl, and five-membered or six-membered aromatic, heteroaromatic, or aliphatic ring; n_{16} is 0 or 1; and X_{17} and X_{18} are independently selected from the group consisting of hydrogen, saturated or unsaturated alkyl, and five-membered or six-membered aromatic, heteroaromatic, or aliphatic ring where each ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;
- 25 (xii) a ketone of formula $-(X_{19})_{n_{19}}-CO-X_{20}$, where X_{19} and X_{20} are independently selected from the group consisting of alkyl and five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where the alkyl and each
- 30

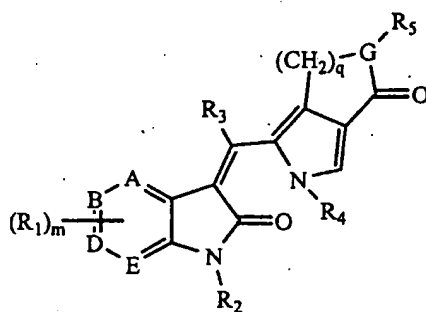
ring is optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring, halogen, trihalomethyl, carboxylate, amino, nitro, and ester; and n19 is 0 or 1;

- 5 (xiii) a nitro group of the formula $-\text{NO}_2$; or
- (xiv) two adjacent R_1 groups together along with two adjacent A, B, D, or E form an optionally substituted five-membered or six-membered aliphatic or heteroaliphatic ring;
- (b) R_2 and R_4 are each independently selected from the group consisting of
- 10 (i) hydrogen;
- (ii) saturated or unsaturated alkyl optionally substituted with one, two or three substituents selected from the group consisting of halogen, trihalomethyl, carboxylate, amino, nitro, ester, and a five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where each ring is optionally
- 15 substituted with one, two or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;
- (iii) an aromatic or heteroaromatic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, and ester; and
- 20 (iv) an aliphatic or heteroaliphatic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, ester, and an aromatic or heteroaromatic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen,
- 25 trihalomethyl, carboxylate, amino, nitro, and ester;
- (c) R_3 and R_5 are each independently selected from the group consisting of
- (i) hydrogen;
- (ii) saturated or unsaturated alkyl optionally substituted with one, two or three substituents selected from the group consisting of hydroxy, halogen, trihalomethyl,
- 30 carboxylate, amino, nitro, ester, and a five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where each ring is optionally

- substituted with one, two or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;
- (iii) an aromatic or heteroaromatic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, and ester; and
- (iv) an aliphatic or heteroaliphatic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, ester, and an aromatic or heteroaromatic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;
- (d) A, B, D, and E are each independently carbon or nitrogen provided that none, one, or, two of A, B, D, and E is nitrogen; and provided that when any of A, B, D, or E is nitrogen, no R_i is attached to said A, B, D, or E;
- (e) G is nitrogen or oxygen, with the proviso that when G is oxygen, R_5 is absent;
- (f) m is 2, 3, or 4; and
- (h) q is 1, 2, 3, or 4; or
- a pharmaceutically acceptable salt thereof.

20

2. A compound of Formula (I):



(I)

25 wherein:

(a) each R_1 is independently and optionally:

(i) hydrogen;

(ii) saturated or unsaturated alkyl optionally substituted with one, two or three substituents selected from the group consisting of:

5 halogen, trihalomethyl, alkoxy, carboxylate, amino, nitro, ester, a five-membered aromatic ring, a five-membered heteroaromatic ring, a five-membered aliphatic ring, a five-membered heteroaliphatic ring, a six-membered aromatic ring, a six-membered heteroaromatic ring, a six-membered aliphatic ring, and a six-membered heteroaliphatic ring;

10 wherein said five- or six-membered ring is optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;

(iii) an aromatic or heteroaromatic ring optionally substituted with one, two, or
15 three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;

(iv) an aliphatic or heteroaliphatic ring optionally substituted with one, two, or three substituents independently selected from the group consisting of:
20 alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, ester, an aromatic ring, and a heteroaromatic ring;

 wherein said ring is optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;

25 (v) an alcohol of formula $-(X_1)_{n1}-OH$ or an alkoxyalkyl of formula $-(X_2)_{n2}-O-X_3$, wherein X_1 , X_2 , and X_3 are independently selected from the group consisting of:

 saturated alkyl, unsaturated alkyl, amide, a five-membered aromatic ring, a five-membered heteroaromatic ring, a five-membered aliphatic ring, a five-membered heteroaliphatic ring, a six-membered aromatic ring, a six-membered heteroaromatic ring, a six-membered aliphatic ring, and a six-membered heteroaliphatic ring;
30

wherein said alkyl group or said five- or six-membered ring is optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, alkoxy, aryl, heteroaryl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester; and

n1 and n2 are independently 0 or 1;

(vi) a halogen or trihalomethyl;

(vii) a carboxylic acid of formula $-(X_4)_{n4}-COOH$ or ester of formula $-(X_5)_{n5}-COO-X_6$, where X_4 , X_5 , and X_6 are independently selected from the group consisting of alkyl, a five-membered aromatic ring, a five-membered heteroaromatic ring, a five-membered aliphatic ring, a five-membered heteroaliphatic ring, a six-membered aromatic ring, a six-membered heteroaromatic ring, a six-membered aliphatic ring, and a six-membered heteroaliphatic ring; and

n4 and n5 are independently 0 or 1;

(viii) an amide or thioamide of formula $-(X_7)_{n7}-NHCOX_8$, $-(X_7)_{n7}-NHCSX_8$, $-(X_9)_{n9}-CONX_{10}X_{11}$, or of formula $-(X_9)_{n9}-CSNX_{10}X_{11}$, where X_7 and X_9 are each independently selected from the group consisting of:

alkyl, a five-membered aromatic ring, a five-membered heteroaromatic ring, a five-membered aliphatic ring, a five-membered heteroaliphatic ring, a six-membered aromatic ring, a six-membered heteroaromatic ring, a six-membered aliphatic ring, and a six-membered heteroaliphatic ring;

wherein said five- or six-membered ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;

n7 and n9 are independently 0 or 1;

X_8 , X_{10} , and X_{11} are each independently selected from the group consisting of:

hydrogen, alkyl, hydroxyl, alkoxy, a five-membered aromatic ring, a five-membered heteroaromatic ring, a five-membered aliphatic ring, a five-membered heteroaliphatic ring, a six-membered aromatic ring, a six-

membered heteroaromatic ring, a six-membered aliphatic ring, and a six-membered heteroaliphatic ring;

wherein said five- or six membered ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, thioalkyl, aryl, heteroaryl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester; or X_{10} and X_{11} taken together form a five-membered or six-membered aliphatic or heteroaliphatic ring or a fused bicyclic ring optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;

(ix) a sulfonamide of formula $-(X_{12})_{n12}-SO_2NX_{13}X_{14}$ or of formula $-(X_{12})_{n12}-NX_{13}-SO_2-X_{14}$, where X_{12} is selected from the group consisting of:

alkyl, a five-membered aromatic ring, a five-membered heteroaromatic ring, a five-membered aliphatic ring, a five-membered heteroaliphatic ring, a six-membered aromatic ring, a six-membered heteroaromatic ring, a six-membered aliphatic ring, and a six-membered heteroaliphatic ring;

where said alkyl or said five- or six-membered ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, halogen, hydroxy, trihalomethyl, carboxylate, amino, nitro, and ester;

$n12$ is 0, 1, or 2; and

X_{13} , and X_{14} are independently selected from the group consisting of:

hydrogen, alkyl, a five-membered aromatic ring, a five-membered heteroaromatic ring, a six-membered aromatic ring, a six-membered heteroaromatic, a three-membered aliphatic ring, a four-membered aliphatic ring, a five membered aliphatic ring, a six-membered aliphatic ring, a three-membered heteroaliphatic ring, a four-membered heteroaliphatic ring, a five membered heteroaliphatic ring and a six-membered heteroaliphatic ring;

wherein said alkyl group is optionally substituted with one, two or three substituents independently selected from the group

consisting of alkyl, alkoxy, aryl, heteroaryl, halogen, trihalomethyl, aldehyde, carboxylate, amino, nitro, and ester;

wherein said five or six membered aromatic ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, aryl, heteroaryl, halogen, trihalomethyl, aldehyde, carboxylate, amino, nitro, and ester;

wherein said five or six membered heteroaromatic ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, aryl, heteroaryl, halogen, trihalomethyl, aldehyde, carboxylate, amino, nitro, and ester;

wherein said three-, four-, five- or six-membered aliphatic ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, aryl, heteroaryl, halogen, trihalomethyl, aldehyde, carboxylate, amino, nitro, and ester; and

wherein said three-, four-, five- or six-membered heteroaliphatic ring is optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, aryl, heteroaryl, halogen, trihalomethyl, aldehyde, carboxylate, amino, nitro, and ester; or

X_{13} and X_{14} taken together form a five-membered or six-membered aliphatic or heteroaliphatic ring or a fused bicyclic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl,

halogen, trihalomethyl, carboxylate, amino, nitro, and ester;

(x) a sulfone of formula $-(X_{15})_{n15}-SO_3H$, where X_{15} is selected from the group consisting of:

alkyl, a five-membered aromatic ring, a five-membered heteroaromatic ring, a five-membered aliphatic ring, a five-membered heteroaliphatic ring, a six-membered aromatic ring, a six-membered heteroaromatic ring, a six-membered aliphatic ring, and a six-membered

heteroaliphatic ring;

where said alkyl or said five- or six-membered ring is

optionally substituted with one, two or three substituents

independently selected from the group consisting of alkyl, halogen,

5 hydroxy, trihalomethyl, carboxylate, amino, nitro, and ester;

n15 is 0, 1, or 2;

(xi) an amine of formula $-(X_{16})_{n16}-NX_{17}X_{18}$, where X_{16} is selected from the group consisting of:

saturated alkyl, unsaturated alkyl, a five-membered aromatic ring, a

10 five-membered heteroaromatic ring, a five-membered aliphatic ring, a five-membered heteroaliphatic ring, a six-membered aromatic ring, a six-membered heteroaromatic ring, a six-membered aliphatic ring, and a six-membered heteroaliphatic ring;

n16 is 0 or 1; and

15 X_{17} and X_{18} are independently selected from the group consisting of:

hydrogen, saturated alkyl, unsaturated alkyl, a five-membered aromatic ring, a five-membered heteroaromatic ring, a five-membered aliphatic ring, a five-membered heteroaliphatic ring, a six-membered aromatic ring, a six-membered heteroaromatic ring, a six-membered aliphatic
20 ring, and a six-membered heteroaliphatic ring;

where said five- or six-membered ring is optionally

substituted with one, two or three substituents independently selected

from the group consisting of alkyl, halogen, trihalomethyl,

carboxylate, amino, nitro, and ester;

25 (xii) a ketone of formula $-(X_{19})_{n19}-CO-X_{20}$, where X_{19} and X_{20} are independently selected from the group consisting of:

alkyl, a five-membered aromatic ring, a five-membered

heteroaromatic ring, a five-membered aliphatic ring, a five-membered

heteroaliphatic ring, a six-membered aromatic ring, a six-membered

30 heteroaromatic ring, a six-membered aliphatic ring, and a six-membered heteroaliphatic ring;

where said alkyl group, five- or six-membered ring is optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, a five-membered aromatic ring, a five-membered heteroaromatic ring, a five-membered aliphatic ring, a five-membered heteroaliphatic ring, a six-membered aromatic ring, a six-membered heteroaromatic ring, a six-membered aliphatic ring, and a six-membered heteroaliphatic ring; halogen, trihalomethyl, carboxylate, amino, nitro, and ester; and n19 is 0 or 1;

- 10 (xiii) a nitro group of the formula $-\text{NO}_2$; or
(xiv) two adjacent R_1 groups together along with two adjacent A, B, D, or E form an optionally substituted five-membered or six-membered aliphatic or heteroaliphatic ring;

(b) R_2 and R_4 are each independently:

- 15 (i) hydrogen;
(ii) saturated or unsaturated alkyl optionally substituted with one, two or three substituents selected from the group consisting of:

halogen, trihalomethyl, alkoxy, carboxylate, amino, nitro, ester, a five-membered aromatic ring, a five-membered heteroaromatic ring, a five-membered aliphatic ring, a five-membered heteroaliphatic ring, a six-membered aromatic ring, a six-membered heteroaromatic ring, a six-membered aliphatic ring, and a six-membered heteroaliphatic ring;

wherein said five- or six-membered ring is optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;

- 25 (iii) an aromatic or heteroaromatic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, and ester; and
30 (iv) an aliphatic or heteroaliphatic ring optionally substituted with one, two, or three substituents independently selected from the group consisting of:

alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, ester,
an aromatic ring, and a heteroaromatic ring;

wherein said ring is optionally substituted with one, two, or
three substituents independently selected from the group consisting of
5 alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, and
ester;

(c) R_3 and R_5 are each independently:

(i) hydrogen;

(ii) saturated or unsaturated alkyl optionally substituted with one, two or three
10 substituents selected from the group consisting of:

hydroxy, halogen, trihalomethyl, alkoxy, carboxylate, amino, nitro,
ester, a five-membered aromatic ring, a five-membered heteroaromatic ring,
a five-membered aliphatic ring, a five-membered heteroaliphatic ring, a six-
membered aromatic ring, a six-membered heteroaromatic ring, a six-
15 membered aliphatic ring, and a six-membered heteroaliphatic ring;

wherein said five- or six-membered ring is optionally
substituted with one, two, or three substituents independently selected
from the group consisting of alkyl, halogen, trihalomethyl,
carboxylate, amino, nitro, and ester;

20 (iii) an aromatic or heteroaromatic ring optionally substituted with one, two or
three substituents independently selected from the group consisting of alkyl, alkoxy,
halogen, trihalomethyl, carboxylate, amino, nitro, and ester; and

(iv) an aliphatic or heteroaliphatic ring optionally substituted with one, two, or
three substituents independently selected from the group consisting of:

25 alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, ester,
an aromatic ring, and a heteroaromatic ring;

wherein said ring is optionally substituted with one, two, or
three substituents independently selected from the group consisting of
alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, and
30 ester;

(d) A, B, D, and E are each independently carbon or nitrogen provided that none,

one, or, two of A, B, D, and E is nitrogen; and provided that when any of A, B, D, or E is nitrogen, no R₁ is attached to said A, B, D, or E;

(e) G is nitrogen or oxygen, with the proviso that when G is oxygen, R₂ is absent;

5 (f) m is 2, 3, or 4; and

(i) q is 1, 2, 3, or 4; or

a pharmaceutically acceptable salt thereof.

3. The compound of Claim 1, wherein:

10 (a) each R₁ is independently and optionally selected from the group consisting of:

(i) hydrogen;

15 (ii) saturated alkyl comprising a branched or straight chain of one to five carbon atoms;

(iii) an aromatic or heteroaromatic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;

20 (iv) an aliphatic or heteroaliphatic ring optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, ester, and an aromatic or heteroaromatic ring optionally substituted with one, two or three substituents independently selected from the group consisting of alkyl, alkoxy, halogen, trihalomethyl, carboxylate, amino, nitro, and ester;

25 (v) an alcohol of formula -(X₁)_{n1}-OH or an alkoxyalkyl of formula -(X₂)_{n2}-O-X₃, where X₁, X₂, and X₃ are independently selected from the group consisting of alkyl comprising a branched or straight chain of one to five carbon atoms and a six-membered aromatic ring; and n1 and n2 are independently 0 or 1;

(vi) a halogen;

30 (vii) a carboxylic acid of formula -(X₄)_{n4}-COOH or ester of formula -(X₅)_{n5}-COO-X₆, wherein X₄, X₅, and X₆ are independently of alkyl comprising a branched or

- straight chain of one to five carbon atoms, and n_4 and n_5 are independently 0 or 1;
- (viii) an amide of formula $-(X_7)_{n_7}-NHCOX_8$, or of formula $-(X_9)_{n_9}-CONX_{10}X_{11}$,
 wherein X_7 and X_9 are each independently alkyl comprising a branched or straight
 chain of five or less carbon atoms, n_7 and n_9 are independently 0 or 1, X_8 , X_{10} , and
 5 X_{11} are each independently hydrogen or alkyl comprising a branched or straight
 chain of one to five carbon atoms or wherein X_{10} and X_{11} taken together form a five-
 membered or six-membered aliphatic or heteroaliphatic ring or a fused bicyclic ring
 optionally substituted with one, two or three substituents independently selected
 from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, amino, nitro,
 10 and ester;
- (ix) a sulfonamide of formula $-(X_{12})_{n_{12}}-SO_2NX_{13}X_{14}$, wherein X_{12} is alkyl
 comprising a branched or straight chain of one to five carbon atoms; n_{12} is 0 or 1;
 X_{13} and X_{14} are independently selected from the group consisting of hydrogen, alkyl,
 and five-membered or six-membered aromatic, heteroaromatic, aliphatic, or
 15 heteroaliphatic ring
 where the alkyl and each ring is optionally substituted with one, two or three
 substituents independently selected from the group consisting of alkyl, halogen,
 hydroxy, trihalomethyl, carboxylate, amino, nitro, and ester; or
 wherein X_{13} and X_{14} taken together form a five-membered or six-membered aliphatic
 20 or heteroaliphatic ring or a fused bicyclic ring optionally substituted with one, two or
 three substituents independently selected from the group consisting of alkyl,
 halogen, trihalomethyl, carboxylate, amino, nitro, and ester;
- (x) a sulfone of formula $-(X_{15})_{n_{15}}-SO_3H$, wherein X_{15} is alkyl comprising a
 branched or straight chain of one to five carbon atoms and n_{15} is 0, 1, or 2;
- 25 (xi) an amine of formula $-(X_{16})_{n_{16}}-NX_{17}X_{18}$, wherein X_{16} is alkyl comprising a
 branched or straight chain of five or less carbon atoms; n_{16} is 0 or 1, and
 X_{17} and X_{18} are independently selected from the group consisting of hydrogen,
 saturated or unsaturated alkyl, and five-membered or six-membered aromatic,
 heteroaromatic, or aliphatic ring;
- 30 (xii) a ketone of formula $-(X_{19})_{n_{19}}-CO-X_{20}$, wherein X_{19} and X_{20} are independently
 selected from the group consisting of alkyl and five-membered or six-membered

- aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring where the alkyl and each ring are optionally substituted with one, two, or three substituents independently selected from the group consisting of alkyl, five-membered or six-membered aromatic, heteroaromatic, aliphatic, or heteroaliphatic ring, halogen, trihalomethyl, carboxylate, amino, nitro, and ester; and n19 is 0 or 1; and
- (xiii) a nitro group of the formula $-\text{NO}_2$; or
 - (xiv) two adjacent R_1 groups together along with two adjacent A, B, D, or E form a five-membered or six-membered aliphatic or heteroaliphatic ring;
 - (b) R_2 , R_3 , and R_4 are hydrogen;
 - (c) A, B, D, and E are each independently carbon or nitrogen; provided that none, one, or two of A, B, D, and E is nitrogen; and provided that when any of A, B, D, or E is nitrogen, no R_1 is attached to said A, B, D, or E;
 - (d) X is nitrogen or oxygen;
 - (e) m is 2, 3, or 4; and
 - (f) q is 1, or 2.

4. The compound of Claim 1, wherein

- (a) each R_1 is independently and optionally selected from the group consisting of:
 - (i) hydrogen;
 - (ii) methyl, ethyl, propyl, ethylene, propylene, or butylene;
 - (iii) an alcohol of formula $-(\text{X}_1)_{n1}-\text{OH}$ or an alkoxyalkyl of formula $-(\text{X}_2)_{n2}-\text{O}-\text{X}_3$, wherein X_1 , X_2 , and X_3 are independently selected from the group consisting of methylene, ethylene, or propylene, and n1 and n2 are independently 0 or 1;
 - (iv) fluoro, chloro, or bromo;
 - (v) a carboxylic acid of formula $-(\text{X}_4)_{n4}-\text{COOH}$, wherein X_4 is selected from the group consisting of methylene, ethylene, or propylene, and n4 is 0 or 1;
 - (vi) an amide of formula $-(\text{X}_7)_{n7}-\text{NHCOX}_8$, or of formula $-(\text{X}_9)_{n9}-\text{CONX}_{10}\text{X}_{11}$, wherein X_7 and X_8 are each independently selected from the group consisting of methylene, ethylene, and propylene, and n7 and n9 are independently 0 or 1;

- X_8 , X_{10} , and X_{11} are each independently selected from the group consisting of hydrogen, methyl, ethyl, and propyl, or wherein X_{10} and X_{11} taken together form a five-membered or six-membered heteroaliphatic ring;
- (vii) a sulfonamide of formula $-(X_{12})_{n12}-SO_2NX_{13}X_{14}$, wherein X_{12} is selected from the group consisting of methylene, ethylene, or propylene, and $n12$ is 0 or 1; X_{13} , and X_{14} are independently selected from the group consisting of hydrogen, methyl, ethyl, propyl, and phenyl, said methyl, ethyl, propyl, and phenyl are optionally substituted with one, two or three substituents independently selected from the group consisting of halogen, hydroxy, carboxylate, amino, nitro, and ester, or wherein X_{13} and X_{14} taken together form a fused bicyclic ring; or
- (viii) two adjacent R_1 groups together along with two adjacent A, B, D, or E form a six-membered heteroaliphatic ring;
- (b) A, B, D, and E are carbon;
- (c) X is nitrogen or oxygen;
- (d) m is 1 or 2; and
- (e) q is 2.

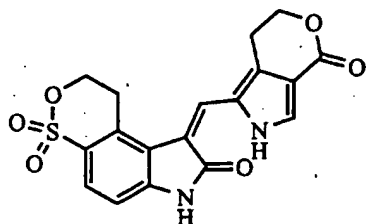
5. The compound of Claim 1, wherein each R_1 is independently selected from the group consisting of methyl, methoxy, 2-hydroxyethyl, phenyl, 2-methoxyphenyl, 3-methoxyphenyl, 4-methoxyphenyl, 4-fluorophenyl, 4-chlorophenyl, 4-bromophenyl, 4-iodophenyl, 3-carboxyphenyl, pyridyl, $-NHC(O)CH_3$, $-C(O)N(CH_3)_2$, $-COOH$, $-SO_2NH(CH_3)$, $-SO_2NH_2$, $-SO_2N(CH_3)_2$, $-SO_2NH(CH(CH_3)_2)$, $-SO_2NH(C_6H_5)$, $-SO_2NH(3\text{-chlorophenyl})$, and $-SO_2N(CH_3)(3\text{-chlorophenyl})$ and m is 1 or 2.

6. The compound of Claim 1, wherein said compound is selected from the group consisting of 4-methyl-2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid methylamide, 2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid methylamide, 1-(4-methyl-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 1-(5-methoxy-2-oxo-1,2-

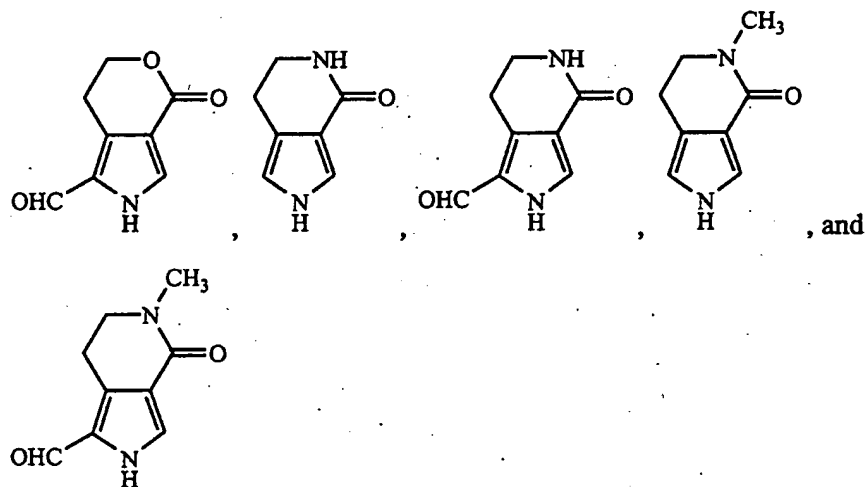
- dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 1-(6-methoxy-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 1-(5-bromo-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 1-(5-chloro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 1-[4-(2-hydroxy-ethyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 1-(2-oxo-5-phenyl-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid amide, 2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid dimethylamide, 2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid isopropylamide, 2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid phenylamide, N-[2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indol-6-yl]-acetamide, 1-(2-oxo-6-phenyl-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 1-(5-fluoro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indole-5-carboxylic acid, 2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indole-6-carboxylic acid, 1-(6-chloro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 1-(6-bromo-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 1-[6-(2-methoxy-phenyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 1-[6-(3-methoxy-phenyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 1-[6-(4-methoxy-phenyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 1-[6-(4-fluoro-phenyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 1-(2-oxo-6-pyridin-3-yl-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 1-[5-(2,3-dihydro-indole-1-sulfonyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2H-

- pyrano[3,4-c]pyrrol-4-one, 1-[5-(3,4-dihydro-2H-quinoline-1-sulfonyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 1-[5-(3,4-dihydro-1H-isoquinoline-2-sulfonyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 1-[5-(5-bromo-2,3-dihydro-indole-1-sulfonyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid (3-chloro-phenyl)-methyl-amide, 1-(6-fluoro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 1-(5-chloro-4-methyl-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 1-(7-chloro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 3-[2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indol-5-yl]-benzoic acid, 3-[2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indol-6-yl]-benzoic acid, 2-oxo-3-(4-oxo-2,4,6,7-tetrahydro-pyrano[3,4-c]pyrrol-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid (3-chloro-phenyl)-amide, 1-(5-bromo-4-methyl-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-6,7-dihydro-2H-pyrano[3,4-c]pyrrol-4-one, 1-(2-oxo-5-phenyl-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one, 1-(2-oxo-6-phenyl-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one, 1-(6-bromo-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one, 1-(6-chloro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one, 2-oxo-3-(4-oxo-4,5,6,7-tetrahydro-2H-pyrrolo[3,4-c]pyridin-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid dimethylamide, 2-oxo-3-(4-oxo-4,5,6,7-tetrahydro-2H-pyrrolo[3,4-c]pyridin-1-ylmethylene)-2,3-dihydro-1H-indole-5-carboxylic acid dimethylamide, 1-[2-oxo-5-(pyrrolidine-1-carbonyl)-1,2-dihydro-indol-3-ylidenemethyl]-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one, 1-[5-(morpholine-4-carbonyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one, 1-[4-(2-hydroxy-ethyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one, 1-(5-methoxy-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one, 1-(5-fluoro-

- 2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one, 1-(5-bromo-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,5,6,7-tetrahydro-pyrrolo[3,4-c]pyridin-4-one, 2-oxo-3-(4-oxo-4,5,6,7-tetrahydro-2H-pyrrolo[3,4-c]pyridin-1-ylmethylene)-2,3-dihydro-1H-indole-5-carboxylic acid, 2-oxo-3-(4-oxo-4,5,6,7-tetrahydro-2H-pyrrolo[3,4-c]pyridin-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid amide, 2-oxo-3-(4-oxo-4,5,6,7-tetrahydro-2H-pyrrolo[3,4-c]pyridin-1-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid methylamide, and



- 10 7. A compound selected from the group consisting of :



- 15 8. A pharmaceutical composition comprising a compound according to Claim 1 and a pharmaceutically acceptable excipient.

9. A pharmaceutical composition comprising a compound according to Claim 3 and a pharmaceutically acceptable excipient.

10. A pharmaceutical composition comprising a compound according to Claim 6 and a pharmaceutically acceptable excipient.
11. A method of treating a disease mediated by a protein kinase in an organism which method comprises administering to said organism a pharmaceutical composition comprising a compound of Claim 1 and a pharmaceutically acceptable excipient.
12. A method of treating a disease mediated by a protein kinase in an organism which method comprises administering to said organism a pharmaceutical composition comprising a compound of Claim 3 and a pharmaceutically acceptable excipient.
13. A method of treating a disease mediated by a protein kinase in an organism which method comprises administering to said organism a pharmaceutical composition comprising a compound of Claim 6 and a pharmaceutically acceptable excipient.
14. The method of claim 13, wherein said organism is a human and said abnormal condition is cancer.
15. The method of Claim 14 wherein said cancer selected is from the group consisting of squamous cell carcinoma, astrocytoma, Kaposi's sarcoma, glioblastoma, lung cancer, bladder cancer, head and neck cancer, melanoma, ovarian cancer, prostate cancer, breast cancer, small-cell lung cancer, glioma, colorectal cancer, genitourinary cancer and gastrointestinal cancer.